Receptor revision and somatic hypermutation- mechanisms of B cell diversification.

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Abbreviations

BCR	B cell receptor
BSA	bovine serum albumin
CDR	complementarity determining region
CFSE	5,6 carboxyfluorescein diacetate, succinimidyl ester
CFA	complete Freund's adjuvant
CSA	chicken serum albumin
DC	dendritic cell
DNA	deoxyribonucleic acid
DNP	dinitrophenyl
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
FDC	follicular dendritic cell
FITC	fluorecein isothyocyanate
FR	framework region
GC	germinal centre
GFP	green fluorescent protein
H	antibody heavy chain
Ig	immunoglobulin
i.p.	intraperitoneally
i.v.	intravenously
Id	idiotype
KI	knock-in
KLH	keyhole limpet haemocyanin
L	antibody light chain
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NP	4-hydroxy-3-nitrophenyl acetyl
OD	optical density
OVA	ovalbumin
PBS	phospate buffered saline
PE	phycoerythrin
Ph-OX	phenyl oxazolone
PNA	peanut agglutinin
PNP	p-nitrophenyl acetyl
QM	quasi monoclonal
RAG	recombination activating gene
RCF	recombinant C fragment from tetanus toxin
RT-PCR	reverse transcription polymerase chain reaction
s.c.	subcutanously
Taq	*Thermus aquaticus*
TD	T dependent
TdT	Terminal deoxynucleotidyl transferase.
TI	T independent
TNP	trinitrophenyl
V(D)J	variable (diversity) joining regions

Abstract

The production of high affinity antibodies is critical for providing effective protection against microbial agents. During the T cell dependent response to antigen, secondary diversification of the B cell receptor occurs. This provides a varied pool of B cells which can be selected and expanded resulting in affinity maturation of the response. This diversification is currently ascribed to somatic hypermutation. Both the molecular basis and the triggers for hypermutation have been studied *in vitro* .

Recently the possibility that another process, receptor revision, might provide an additional means of diversification has emerged. Receptor revision involves the secondary rearrangement of the B cell receptor in the periphery, as yet no purpose for this process has been revealed. Experiments are described which investigate a potential contribution of receptor revision to affinity maturation. A pure population of transgenic B cells are adoptively transferred into chimaeric hosts and the affinity maturation of these cells is monitored in isolation. The development of the transfer system is documented and results presented which show no evidence for a role for receptor revision in affinity maturation.

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Chapter 1 Introduction

1.1 Antibodies and the adaptive immune response.

Antibodies have 2 main functions: to recognise and bind pathogens and to recruit other cells and molecules of the immune system to destroy them. They have the capacity to bind strongly to an almost infinite number of foreign antigens, an ability that results from the complex nature of the genetic locus encoding these soluble proteins. The finding that B cells were responsible for the soluble factors that combat disease was a landmark discovery (reprinted in (1)). The further finding that individual B cells express only a single specificity, and that clones of T and B cells are expanded by antigen contact with receptors on the cell surface, paved the way for the study of the adaptive immune response (2).

The ability to secrete molecules that specifically recognise a pathogen is a feature of the adaptive immune response. Adaptive immune responses are a feature of vertebrates, and rely on the split nature of B and T lymphocyte receptor genes. When randomly recombined and expressed as protein these genes give rise to a highly diverse B and T lymphocyte repertoire (3). The development of the adaptive immune system is thought to have occurred subsequent to the arrival of the recombination activating genes (RAG1 and 2) in the genome, soon after the divergence of jawed and jawless vertebrates in evolution (4).

The RAG genes are proposed to have entered the vertebrate genome through a transposition event as they have striking similarities to a transposable element (4). RAG recombinase allows the joining of antibody gene segments to

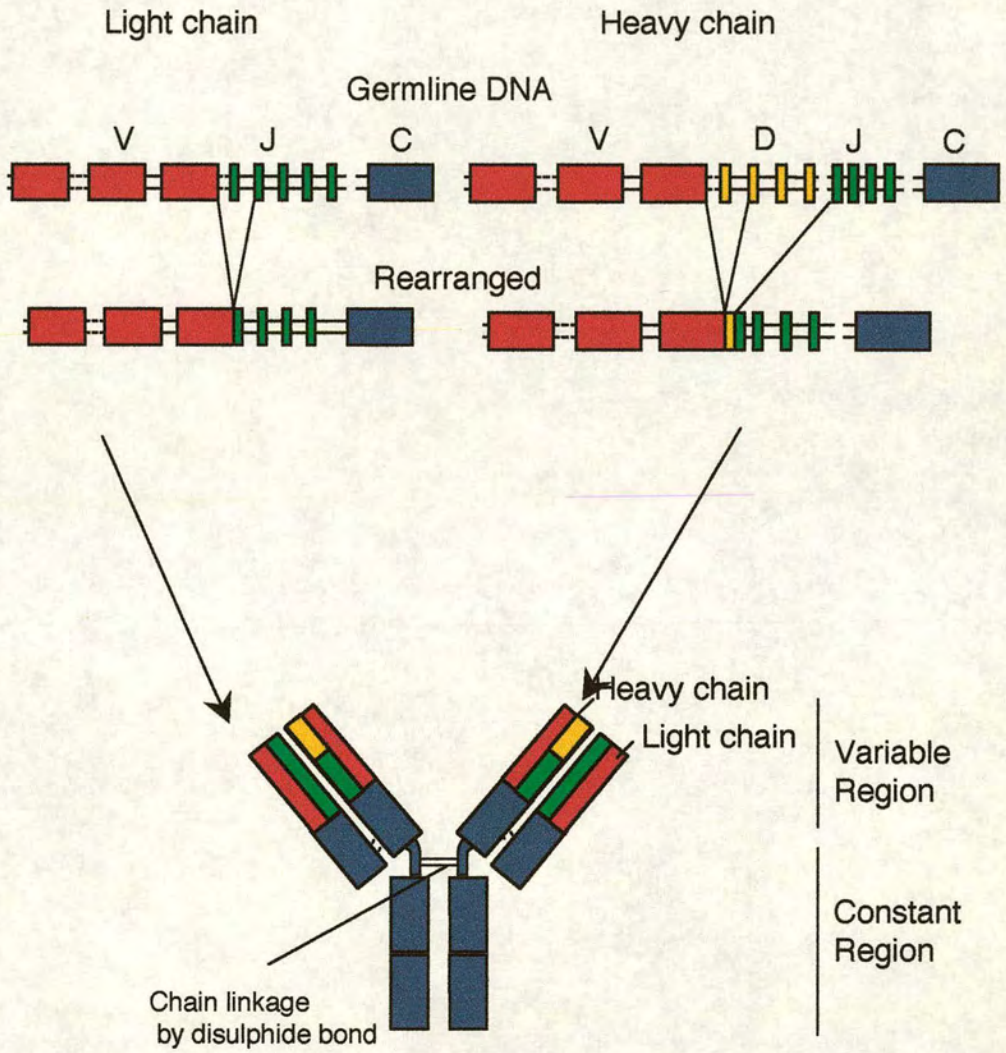
form a gene that can be transcribed and translated into functional protein. Gene duplication events resulted in the generation of multiple gene segments in the antibody coding locus. The incorporation of RAG genes into the vertebrate genome enabled harnessing of gene segments into a mechanism, which allows the generation of diversity required for the adaptive immune system.

1.2 Generation of antibody diversity

Antibody diversity is essential to ensure that the body can combat the multitude of antigens presented to it. In humans the antibody repertoire consists of about 10^{11} different antibody molecules. This diversity is generated through a variety of mechanisms as the B cell develops in the bone marrow (reviewed in (3)). B cells develop from a putative common lymphoid progenitor, to pro-B, pre-B and the immature B cell stages (5), during which time antibodies are generated and expressed on the cell surface as B cell receptors (BCR).

Antibody molecules are made up of two protein chains: one heavy (H) chain and one light (L) chain (of which there are 2: κ and λ). Each chain consists of a constant region, which is highly conserved, and a variable region, which is poorly conserved. Variable regions make contact with antigen, while constant regions provide the structure, and in the case of the heavy chain, perform effector functions. Within the variable regions there are three regions that are hypervariable, these are the points of contact with antigen and are termed complementarity determining regions (CDRs). Intervening parts of the variable region are structural and are termed framework regions (FRs) (Figure 1.1).

Figure 1.1 Antibody gene rearrangement and antibody structure



The simplified structure of an immunoglobulin molecule and its generation from rearranged antibody gene segments

The variable regions of heavy and light chains, which are most important for antigen binding, are encoded by either three or two gene segments respectively. These segments are called the variable (V), diversity (D), and joining (J) genes. Before the variable region can be transcribed, these gene segments must be placed adjacent to each other in a recombination event. There exists a selection of V, (D) and J segments, one of each of these is chosen at random to make up a functional variable region (3)(Figure 1.1).

V, D and J segments are flanked by recombination signal sequences (RSSs), which consist of a heptamer sequence adjacent to the V, D or J region followed by either a 12 or 23 base pair (bp) spacer region, followed by an A-T rich nonamer sequence. To ensure correct rearrangement of V genes one RSS can only combine with another if they have different length spacers, the so called 12/23 rule. At the time of recombination, extra variation termed junctional variation is added in the form of extra nucleotides: P nucleotides are added in a template dependent fashion, whereas N nucleotides are non-templated and are inserted by terminal deoxynucleotidyl transferase (TdT). V(D)J rearrangement is dependent on both RAG1 and RAG2 (6). Both of these genes are conserved between species that undergo V(D)J recombination but are unrelated to each other. Mice that lack either of these genes are unable rearrange immunoglobulin (Ig) or T cell receptor genes and as a result have no T or B lymphocytes (7).

Following the rearrangement of antibody V genes, a further level of diversity is added when the pairing of heavy and light chains to form functional antibody occurs. This is called combinatorial diversity, most heavy chains can pair with most light chains and different combinations may provide different

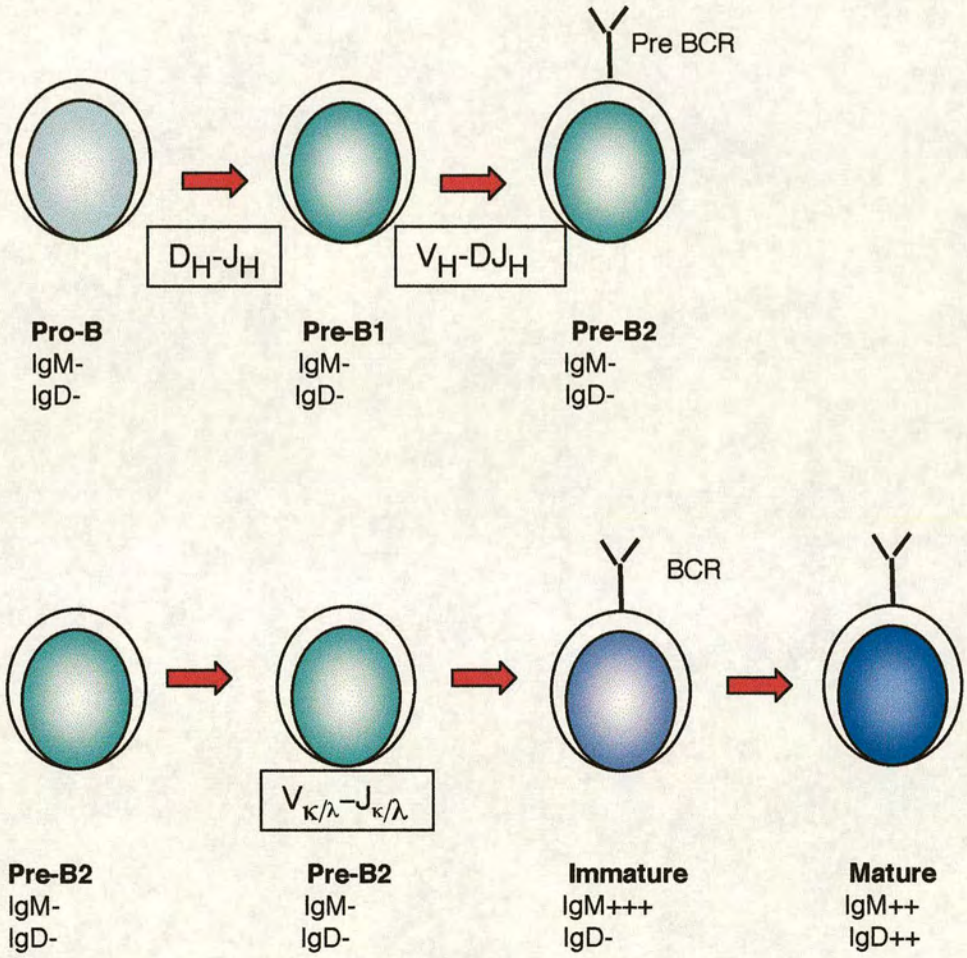
specificities. Once a functional antibody molecule has been produced by a B cell, the recombination process is turned off so that each B cell only expresses a single antibody specificity, a process known as allelic exclusion (reviewed in (8)).

1.3 Selection of the B cell repertoire

Checkpoints exist in the development of B cells, to ensure that the molecular processes described above have occurred correctly. Successful heavy chain rearrangements are tested at the early pre-B cell stage by pairing with the surrogate light chain (reviewed in (9)). Expression of this pre-BCR at the cell surface signals an end to heavy chain rearrangement, and leads to a phase of clonal expansion, followed by rearrangement of the light chain (10-12) (Figure 1.2). The failure to complete these stages successfully results in developmental arrest followed by death by apoptosis (13).

Immature B cells with successful heavy chain and κ or λ light chain pairings then undergo negative selection to remove cells that bind self-antigen. There are 3 possible fates for autoreactive cells in the bone marrow, they can be eliminated by apoptosis (deletion) (14, 15), be functionally inactivated (anergised) (16), or they may be given a second chance at antibody rearrangement, this is known as receptor editing (17). Deletion of autoreactive B lymphocytes was detected in bone marrow chimaeras by injecting transgenic B cells expressing an anti self specificity (anti-H-2K^{K}). Autoreactive B cells were absent in mice expressing H-2K^{K} but present in non-H-2K^{K} mice (15). In contrast immature B cells are made anergic if they encounter soluble antigen.

Figure 1.2 Stages in B cell development in the bone marrow.



B cell development from less mature to mature proceeds from left to right. gene rearrangements are shown in boxes and the Ig phenotype is shown below each cell. Adapted from ref. 208.

They are then unable to respond to subsequent antigen exposure, due to a partial block in signal transduction, however, this may be reversed if the cells are given help from T cells (16).

The factors involved in the cell fate decision of immature B cells are unknown, however, strength of signalling through the BCR, and surface BCR expression levels may be important (18-20). Mechanisms may also be able to compensate for each other. In mice with impaired apoptosis due to the over expression of the anti apoptotic molecule BCL-2, receptor editing was found to be increased (21). Also mice over expressing another anti-apoptotic molecule BCL-X_L were found to compensate for lack of apoptosis by increasing the levels of anergy induced (22).

Receptor editing is of particular interest in the context of this thesis as it is mechanistically related to receptor revision, which is secondary rearrangement of the BCR in the periphery. However, the 2 have different consequences for the fate of the B cell. Receptor editing is described in greater detail below.

1.3.1 Receptor editing

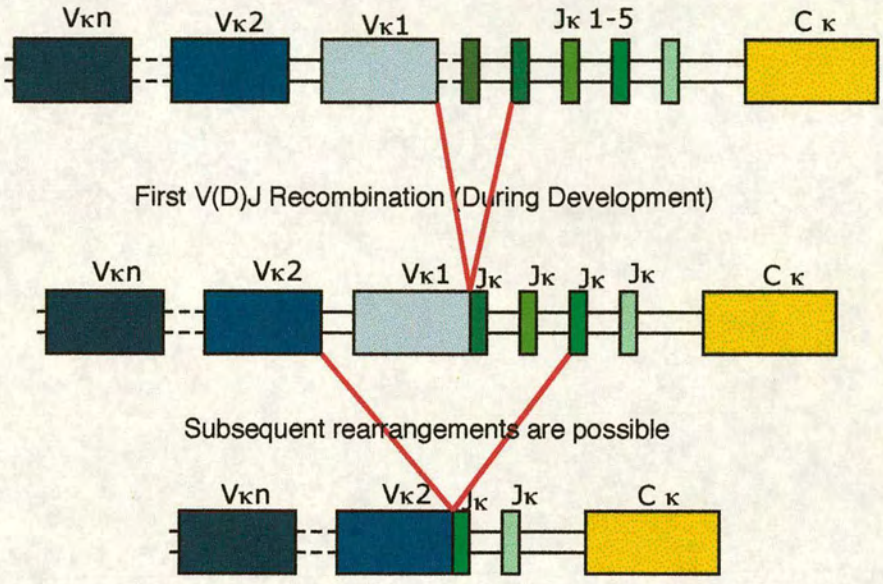
RAG expression in the bone marrow is coincident with the rearrangement of heavy and light chains in pro and pre-B cells. If immature B cells in the bone marrow recognise self-antigen they can maintain expression of RAG 1 and RAG 2 and alter their BCR through a process known as receptor editing (17). This phenomenon was first detected in the bone marrow of transgenic mice carrying autoreactive BCRs (17, 23, 24). Tiegs *et al.* (17) used a mouse with a transgenic BCR specific for H-$2K^{k,b}$ major histocompatibility complex (MHC) molecules (3-

83 transgenic mouse) to investigate events involved in tolerance in the bone marrow. Upon encountering a membrane bound K^b or K^k protein, some immature B cells modified their receptors by RAG dependent secondary rearrangement. Elevated levels of RAG 1 and RAG 2, along with the Ig rearrangement excision products, were detected using a PCR assay. This resulted in expression of a non-transgenic antibody light chain, which was detected by fluorescence activated cell sorting (FACS). They coined the term receptor editing. It had previously been believed that light chain rearrangement was blocked by the expression of a functional antibody on the surface, but discovery of circular DNA excised by nested κ rearrangements suggested that light chain rearrangement could continue (25).

Receptor editing is now a well-studied phenomenon and has also been shown to occur at the heavy chain locus (26, 27) and in B cell development in normal, non-transgenic mice (28). Its role in the generation of the repertoire in mice is considerable. Casellas *et al.* (29) found that about 25% of all antibody molecules are produced by gene replacement by examining the make up of expressed antibody chains in a mouse that contains one pre-rearranged κ chain and a second allele that has an easily detectible human Cκ constant region. This suggests that the receptor editing process plays a major role in the shaping of the antibody repertoire.

To facilitate receptor editing, primary rearrangements at the κ locus are well suited for further recombination as the rearranged VκJκ is flanked by unrearranged Vκ and Jκ segments (Figure 1.3).

Figure 1.3 Schematic representation of receptor editing at the light chain locus.



Receptor revision at the kappa light chain locus. Following RS recombination lambda can also be rearranged and a similar phenomenon has been observed for the heavy chain. (see text for details)

In the heavy chain things are somewhat more complicated, as the presence of the D segment means that there is no longer a RSS present to allow RAG recombinase to recognise the DNA (30). However, receptor editing can still occur at the heavy chain locus due to the presence of RSS-like sequences embedded in the coding region, which allow secondary V(D)J rearrangement to displace the V gene segment from the DJ segments and replace it with a new one (31, 32). Secondary D to J joining can also replace primary rearrangements by deletion or inversion (30).

1.4 T dependent and T independent antigens

B cells that have successfully rearranged non-autoreactive BCRs in the bone marrow, upregulate IgM and are exported to the periphery where they are ready to encounter antigen. Histological studies have shown that B cells interact with specific T cells at the border of the B cell follicle within 2 days of immunisation (33). However the site of initial antigen encounter by naïve B cells is still unclear. A subset of dendritic cells (DCs) that captured antigen rapidly after immunisation has been discovered, which may be involved in activation of T and B cells at the follicular border (34).

The type of response generated by B cells is dependent on the nature of the antigen encountered. B cell responses can be broadly divided into those that are T cell dependent (TD) or T cell independent (TI) based on the response of B cells in *Nude* mice, a congenitally athymic strain that do not have any T cells. Antigens that elicit an antibody response in *Nude* mice are termed TI, those that do not are termed TD.

TD antigens are protein based; they can be processed and presented by B cells as MHC class II bound peptides. These are recognised during cognate interaction with $CD4^+$ T cells (35), which then provide help to B cells in the form of secreted cytokines and molecular costimulation (through CD40) resulting in B cell activation. B cell activation in a T dependent fashion initiates a host of characteristic processes (reviewed in (36)): germinal centres (GC) form, somatic hypermutation is induced and affinity maturation of the antibody response ensues. The development of B cell memory has also been reported to be a TD phenomenon (37), however this may not always be the case (38).

TI antigens are carbohydrate and lipid based. They can be divided into 2 subsets – TI-1 and TI-2, which have different characteristics. TI-1 antigens are typified by bacterial lipopolysaccharide (LPS), which contains multiple repeating epitopes. They are intrinsically mitogenic for B cells and can induce a polyclonal, non-antigen specific response, as they do not necessarily activate through the BCR. However, antigen specific responses to TI-1 antigens are also possible. TI-2 antigens also possess repeating epitopes, typified by bacterial capsular polysaccharides, and can hence also activate B cells in the absence of T cells. However, they are different from TI-1 antigens in that the response to them is always antigen specific. Activation by TI-2 antigens is dependent on the molecule Bruton's tyrosine kinase (Btk) (39). During B cell activation with a TI-2 antigen small numbers of membrane bound antigen clusters induce membrane association of Btk molecules, which induce a calcium flux, recruiting transcription factors and resulting in activation of the B cell. Hence antigen specific immune responses can be elicited to TI-2 antigens (39).

Model TI-2 antigens have carbohydrate backbones and cannot be processed and presented to T cells, however some proteinaceous viruses have repeating epitopes allowing TI activation but may also be processed and presented by B cells recruiting T cell help. This is exemplified in mice infected with certain viruses e.g. rotaviruses. Although infection of mice with viruses normally elicits a TD response, in the absence of αβ T cells a class switched TI response can be elicited (40). Antibody responses of the IgG2a, IgG2b or IgA but not IgG1 isotypes were detected. Help for B cells to class switch in this case is proposed to come from cytokines secreted by activated DC, natural killer (NK) cells or macrophages. Although the classification of TD and TI may be useful for model antigens, it appears that real pathogens, which interact with the immune system differently, may not be as easily classified.

1.5 Isotype switching

The Ig heavy chain can alter its constant region by rearrangement, a process known as isotype switching. Switching to different isotypes results in different effector functions for antibodies and hence is useful in enabling the immune system to use the most effective mode of action against different pathogenic targets. Isotype switching is typically associated with TD immune responses, however, limited switching can also occur in response to some TI antigens.

There are 5 classes of immunoglobulins in rodents and man: IgM, IgD, IgG, IgE and IgA. Naïve B cells express IgM and IgD on their cell surface, these are expressed by default and are generated by alternative splicing of the same mRNA transcript. The expression of other classes requires a recombination event similar to that described for the generation of antibody V regions,

however, the process is RAG recombinase independent. Although switch recombination signals are not as well defined as RSS sequences, a 4 bp sequence has been implicated (41). As the intervening piece of DNA is excised during isotype switching, a cell can switch again but only to isotypes encoded by downstream DNA (42).

IgM is the first isotype secreted in response to infection, it is primarily involved in complement activation but also has a neutralising role. Isotype switching results in the secretion of other antibody isotypes. In the mouse there are 4 subclasses of IgG (IgG1, IgG2a, IgG2b and IgG3). The IgG subclasses neutralise bacterial toxins and viruses, activate complement, and opsonise extracellular pathogens for phagocytosis by neutrophils and macrophages. IgA antibodies are prominent at mucous membranes and may be responsible for protective immunity – the prevention of systemic infection by forming a barrier at susceptible sites. IgE triggers degranulation of mast cells and basophils and is hence associated with allergic responses, it is also prevalent in the gut upon parasitic infection (reviewed in (43)).

Class switching is dependent on B cell activation by antigen, but also in the context of a TD response, signals through CD40 are required (44-46). Switching to different isotypes is also influenced by the cytokines secreted by T cells and other cell types (47-49). For example, switching to IgG1 is associated with Th2 responses as switching to γ1 occurs in response to IL4, which is secreted by these cells. Cytokines exert their influence by inducing the transcription of RNA molecules called sterile transcripts, which are required for isotype switching to occur (50). Transcription of these small RNA molecules may regulate accessibility to the DNA hence allowing switch recombination to

occur. Switching occurs about 6 days after activation in a TD response, just after GC formation and the initiation of somatic hypermutation (51).

In the mouse, model TD antigens elicit robust antibodies of all isotypes, whereas model TI-1 antigens induce only IgM and IgG3 responses. The response elicited by TI-2 antigens is less easily defined. The finding that TI-2 antigens can provide a TI antibody response does not mean that T cells cannot play a permissive role as this class of antigens can sometimes recruit T cell help. In normal mice stimulation through CD40 has been shown to synergise a TI–2 response *in vitro* (52) and *in vivo* (53). This indicates that although that B-T cell interaction is not a requirement for isotype switching in an immune response to TI-2 antigens, that T cells may influence the response.

In the absence of the possibility of costimulation through CD40 in CD40 deficient (44) and CD154 deficient mice (54) TI-2 antigens can still elicit antibody responses of all classes. Isotype switching in this case is elicited by cytokines secreted by other cell types. It is now known that multiple cell types that participate in the immune response can secrete cytokines. Specifically IL-4, IFNγ and TGF-β can be synthesised by both T and non-T cells. Mast cells can secrete IL-4, NK cells are a source of IFNγ (47); , TGF-β can be made by B cells and macrophages (48).

Class switched antibodies are monomeric (with the exception of IgA which is dimeric). In contrast IgM, the first antibody class to be secreted is pentameric. In order to make up for the shortfall in avidity of monomeric antibodies, B cells undergo the process of affinity maturation (55). This results in the generation of high affinity class switched antibodies.

1.6 Affinity maturation

Serum antibody elicited following immunisation with TD antigen increases in affinity for antigen during the course of an immune response (56-58). The secondary B cell repertoire, which is derived from the B cells triggered in the primary response, also consists of cells with improved antigen binding characteristics. This improvement in the affinity of BCRs and secreted antibody for antigen is known as affinity maturation and is dependent on somatically induced variation of antibody genes, and the subsequent selection of high affinity antibodies.

Somatic hypermutation has long been known to contribute to affinity maturation and is a well-studied phenomenon, although many interesting questions about it still remain. During the process of somatic hypermutation point mutations, but also rare deletions and insertions are introduced into the variable region DNA of the heavy and light chains. These are responsible for the alteration of affinity, due to amino acid substitutions that subtly change the shape of the protein, affecting antigen binding sites. The mutation process is very effcective at creating antibodies with altered affinities for antigen. Increases in affinity ranging from ten to one hundred fold can result from as few as two amino acid substitutions in a 100 residue V region (59-61). However, the majority of expressed mutations in B cells do not confer higher affinity and may even result in loss of binding (62). Until recently somatic hypermutation in the GC was thought to be the only mechanism leading to somatic receptor diversification and affinity maturation. Receptor revision a recently reported means of inducing somatic variation may also play a role.

Variation introduced into antibody genes during an antigen dependent response is generated randomly in B cells. Following this generation of diversity, a small number of high affinity antigen binding B cells, improved in somatic events are selectively amplified. This is due to the improved binding of their receptors to the antigen presented in the GC on follicular dendritic cells (FDCs) and hence, more efficient recruitment of T cell help. The selection of high affinity variants results in an improved antibody response and is the basis of affinity maturation.

1.7 The germinal centre – the site of affinity maturation.

GCs are distinctive areas of B cell proliferation that develop in B cell follicles during the first week of a TD antigen response. The GC reaction has been proven to be pivotal in the maturation of primary antibody responses and the development of memory B cells through studies of human and mouse GCs.

The first investigations into the role of GC in affinity maturation were made by MacLennan and Gray (63). The strong correlation between the kinetics of GC formation and the induction of somatic mutation, led them to suggest that affinity maturation took place in this compartment. Direct evidence that mutation occurs in GCs was provided by amplifying and sequencing V regions of GC B cells. These cells were either microdissected from individual GC (64) or were isolated on the basis of their affinity for peanut agglutinin (PNA) – only GC B cells up regulate the sialic acid modification that this lectin recognises (65). In this study mutations in response to the hapten 2-phenyl-oxazolone (Ph-OX) were detectable in GC B cells from day 12, whereas mutations did not appear in the non-GC B cells until 4 weeks after immunisation. Results from these

experiments prove that GCs are the site of affinity maturation and the location of most highly mutated B cells.

Despite the fact that most mutation occurs in GC there exists some evidence for the occurrence of V region mutation outside the GC, but at a lower level. Affinity maturation has been shown to occur in lymphotoxin α deficient mice, which fail to form GCs (66). Mutation was noted in response to 4-hydroxy-3-nitrophenyl (NP), and on continued stimulation with high doses of antigen somatic mutations were noted, although at only 30% of the total number of wild type (wt) mutations. A similar phenomenon was found in mice transgenic for mCTLA4-Hγ1, which also fail to form GC. These mice have somatic mutations in hybridomas generated from B cells but at a decreased level (67). Although these results provide evidence for somatic mutation occurring outside the GC they are still consistent with GCs being the principal site of activation of the process under normal physiological conditions.

1.8 The germinal centre reaction

In response to model protein antigens, GCs tend to peak in numbers at around 10 days and persist for around 30 days (68). In addition to cycling B cells, GCs also contain a population of $CD4^+$ T cells that are antigen specific (69); and a supporting stromal network of FDCs. In addition, there is a substantial number of macrophages that deal with the large amount of apoptotic cell death that results from negative selection of low affinity mutants generated there. These macrophages are termed tingible body macrophages as apoptotic bodies are visible within them making them morphologically distinctive.

As mentioned, signals from T helper cells are required for GC initiation, however, later signals from GC T cells are also required for GC maintenance. MacLennan's group found that in the unusual case of an NP specific site directed transgenic mouse, GC could be initiated by the TI-2 antigen NP-Ficoll (70). However these GC rapidly disintegrated 5 days after immunisation, indicating that GC maintenance is dependent on T cell signals. In particular, CD40 on B cells and its ligand CD154, which is induced on T cells following activation, have been shown to be critical for the development of GC and the induction of somatic hypermutation. Mice defective in either of these molecules do not form GCs and have severely diminished IgG responses to TD antigens (44, 45). Treatment of pre-immunised mice with anti-CD154 or anti-B7-2 completely inhibits GC formation. Later in the response treatment with anti-CD154 but not anti-B7-2 results in the disintegration of preformed GC indicating that signals through CD40 are required for both the initiation and maintenance of GC (71).

In addition to CD154 and B7-2 the inducible costimulatory molecule (ICOS), expressed by activated T cells is involved in GC development. ICOS deficient mice have few GC and decreased antibody production. Conversely mice that over express the ligand for ICOS (B7RP-1) have enlarged lymphoid tissues that have plentiful GCs and plasma cells (72).

Following antigen specific B and T cell interaction in the primary follicles. B cells collect in FDC networks where GC founder B cells undergo rapid proliferation. FDCs retain antigen-antibody complexes that are critical for the selection and survival of high affinity B cell that have undergone mutation of their V regions. GC develop to their fully formed state in around one week.

They can be seen to be polarised morphologically, into a light zone and a dark zone. Centroblasts, B cells in the basal dark zone of the GC proliferate extensively with a division rate of six hours, giving rise to centrocytes, which migrate into the apical light zone (73). It is during the proliferative stage in the dark zone that somatic hypermutation occurs. Subsequently selection of affinity-matured mutants occurs in the light zone.

It has been proposed that cyclic re-entry from light zone to dark zone results in the high rate of mutations accumulated (74). Centrocytes, B cells in the light zone, express low levels of the anti apoptotic molecule BCL2 and high levels of the pro-apoptotic molecule FAS (75). This renders these cells prone to apoptosis unless they are positively selected. In the absence of FAS on B cells clonal selection in GC and the subsequent generation of the memory pool is impaired (76).

The survival of GC centrocytes requires signals through the BCR from antigen on the surface of FDCs and from antigen specific T cells through CD40. Somatically mutated centrocytes are selected according to the affinity of their surface immunoglobulin (sIg) for antigen expressed on the surface of FDCs. Cells with lower affinity for antigen are out-competed for FDC binding and die as they are not positively selected. This phenomenon has been mimicked *in vitro*. It has been shown that the tendency of tonsillar B cells to undergo apoptosis can be delayed by crosslinking their sIg using anti Ig immobilised on solid supports, imitating the interaction with FDCs (77). Later in the selection process a signal for long-term survival is provided by T cell help in the form of CD40 ligation (77). Any autoreactive B cells which may have been generated by

the mutation process will not receive this second signal and will die preventing any deleterious effects of the high level mutation process (Figure 1.4).

1.9 Somatic hypermutation

1.9.1Discovery of somatic hypermutation.

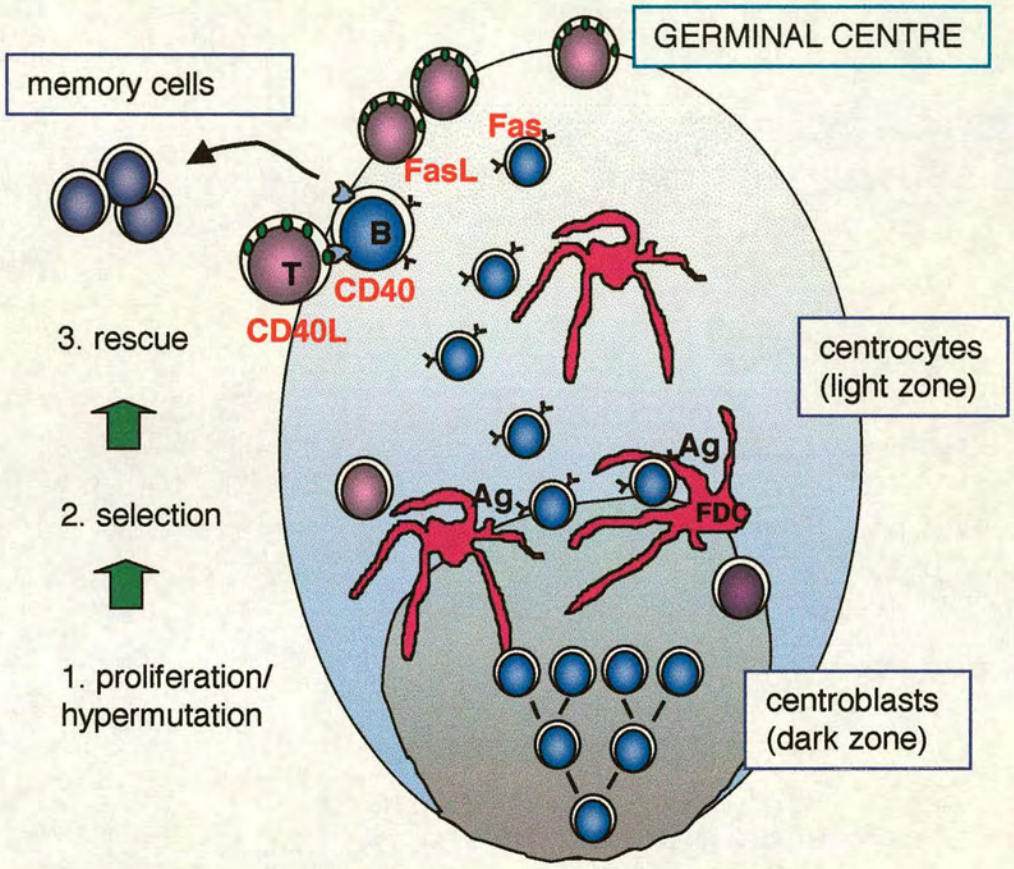
Somatic hypermutation of B cells plays a key role in affinity maturation . The first suggestion that somatic mutation could enrich the diversity of antibodies was made by Burnet (2), as part of his clonal selection theory. These ideas were clarified by Lederberg (78) who presciently suggested that

> 'The hypermutability of a patch of DNA may be a specially evolved solution'

The first study to really show that this indeed may be the case was from Weigert *et al.* (79). They sequenced DNA from plasmacytomas expressing the same Vλ1 gene and found that few of them were the same. They hypothesised that somatic diversification by spontaneous mutation resulted in the mutants discovered. The final evidence for the introduction of point mutations into antibody V genes, was presented simultaneously by several groups in 1981. Specific mouse strains and specific antigens (the response to which had been well characterised) were employed, allowing clear distinction of the sequence of mutated V genes from that of the germline (80-82).

It has been since been estimated, by analysing V region DNA sequences at various time points in the immune response and estimating the number of cell divisions which occurred in that time, that the mutation rate in the primary response is 10^{-5} mutations per bp per generation and 10^{-3} per bp per generation in the secondary response (83).

Figure 1.4 Germinal centre reaction



Mutation of proliferating centroblasts in the dark zone is followed by migration to the light zone and selection on the basis of affinity for antigen.
T cells provide a rescue signal for cells that bind foreign antigen, autoreactive cells which fail to receive this signal die. (see text for further details)

This rate is significantly higher than the background mutation rate of 10^{-12} mutations per bp per generation. In fact, it has been hypothesised that if the rate of mutation was any higher that selection of high affinity mutants would be compromised (84).

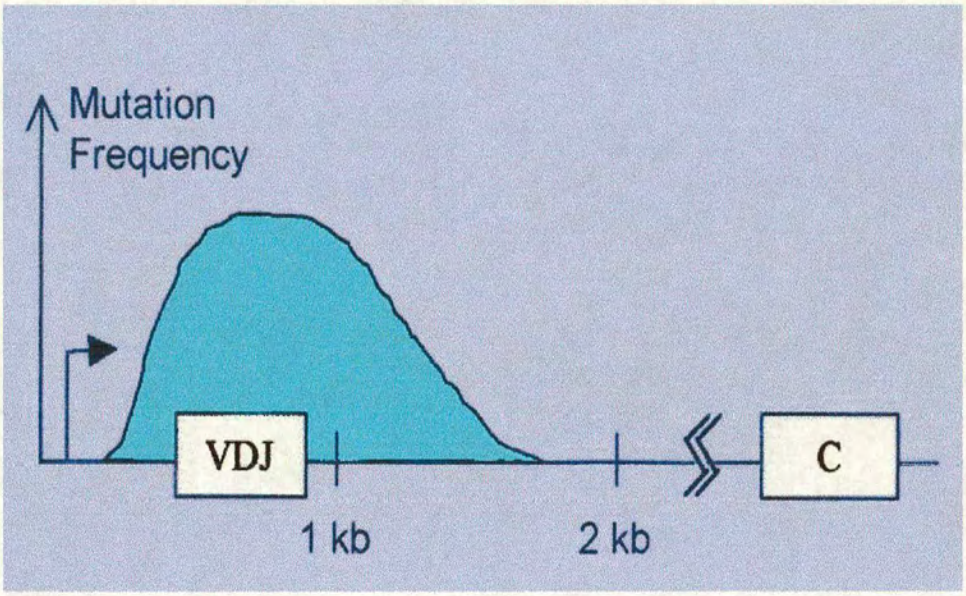
1.9.2 Characteristics of somatic hypermutation.

Somatic hypermutation is an amazing phenomenon; no comparable event occurs elsewhere in the genome. Indeed, mechanisms are in place in most cell types to prevent mutation of any sort occurring. Although the timing and location of the process have been elucidated, the molecular mechanisms and *in vivo* triggers as yet remain unexplained. The analysis of the intrinsic characteristics of somatic hypermutation is important as it gives an insight into the molecular mechanisms involved in the mutation process.

The most intriguing feature of somatic hypermutation in B cells is the specificity of this high rate mutation (4% in mammals), which is many orders of magnitude higher than the spontaneous rate, to the V region only (85). However, the nature of the mutations themselves is also of interest: they are normally nucleotide substitutions, with rare inversions or deletions. There is a preference for transitions over transversions and there is a coding strand bias, also different nucleotide sequences have varied mutability resulting in intrinsic hot and cold mutation spots.

The 5′ boundary of mutation is within the leader intron of the Ig V region gene. Mutations extend over 1.5kb with a slow decline in frequency beyond the V-J join and there is no defined 3′ boundary (85) (Figure 1.5). Mutations are mainly seen to occur in the three CDRs, which are the contact areas for antigen.

Figure 1.5 Distribution of mutations over the antibody coding region.



Mutations are introduced over the entire V region DNA, starting at the leader sequence and continuing over the rearranged V region into the downstream intronic region. (From ref. 83)

Mutations in CDRs are more likely to affect antigen binding affinity and are consequently more likely to be selected explaining the prevalence of mutations in these regions. Approximately 30% of total mutations are silent, these being distributed equally between CDRs and FRs as they are not selected for, indicating that the mutation mechanism itself does not discriminate between these two regions (86)

The mutation process was at first thought to occur randomly along the V region. However it was noticed that certain sequences of DNA carried mutations more frequently than others. These regions are called hot and cold spots and their discovery has given a further insight into process. The question arose as to whether hotspots were due to antigenic selection, or due to local nucleotide sequence. The occurrence, however, of frequently repeated silent mutations, which are not selected for, suggested that intrinsic hotspots of mutation might exist (87).

Nucleotide substitution preferences have been determined by analysing somatic mutation in passenger transgenes, which are not translated due to the presence of translational termination codons within the coding region (upstream, downstream and non-productively rearranged alleles can also be used for the same purpose). For this reason there is no antigenic selection and all mutations can be seen in an equal light, allowing intrinsic hotspots to be distinguished from those that are selected. Data shows that mutations are not random for either base substitution preferences or distribution. They also showed that the effect of skewing by antigenic selection can be considerable; key mutations that increase affinity were frequently found (88, 89).

The mutation mechanism, as well as displaying preference for certain sequences, also displays a preference for the type of mutation. A bias for transitions (Purine (A or G) to Purine or Pyrimidine (T or C) to Pyrimidine) over transversions (Purine to Pyrimidine or vice versa) is an intrinsic property of the mutation mechanism with a 59% transition frequency as opposed to 33% frequency anticipated on a random basis (90, 91). There also exists clear evidence of a strand bias to the mutation process; on the coding strand Gs and As are mutated at a higher frequency than on the non-coding strand. (92). This strand bias made it seem unlikely that an error prone DNA polymerase was involved (85), as this would target both strands equally due to the nature of the replication process. However, the recent discovery that mutation cannot occur in the absence of DNA polymerase iota in a Burkitt's lymphoma cell line may conflict with this (101).

The intrinsic features of somatic mutation discussed above have helped to shed some light on the mechanism of mutation. These, along with the now known cis-acting sequences required for the process, have led to the development of possible models to explain what is happening and how it happens.

1.9.3 Cis-acting DNA sequences

The elucidation of the mechanism of somatic hypermutation has been aided by the investigation of the local regions of DNA, which, acting in cis, are required for the process. Following the discovery by Hackett (93) that chromosomal location was of no importance, the requirement for V region DNA itself has been investigated. It was shown by replacing the V region with various

heterologous sequences including human *β globin* or prokaryotic *gpt* and *neo* genes, without affecting somatic mutation, that the V region coding DNA sequence is not of importance (94). This study demonstrates that non-evolutionarily selected sequences can still display features of mutation similar to that which occurs in the V region.

The requirement for the V region promoter has been investigated, as a link between mutation and transcription was suspected. This was considered due to the lack of mutation in unproductively rearranged and non transcribed V genes, which had been allelically excluded. It has been shown that the Vκ promoter can be replaced by the β globin promoter with little change in the resulting mutation frequency, indicating that the promoter does not target the mutation mechanism to the V region (95). It has since been shown that perhaps all that is required for mutation is an active promoter, resulting in transcription of the gene, and that specificity is provided in another fashion (85). More recently mutation of the heavy chain has been shown to correlate with transcription (96). In this study passenger transgenes were placed under the control of either an RNA polymerase I or II promoter or no promoter at all. The mutation frequencies correlated with the levels of transgene-specific pre-mRNA expressed in GC B cells isolated from the transgenic mice.

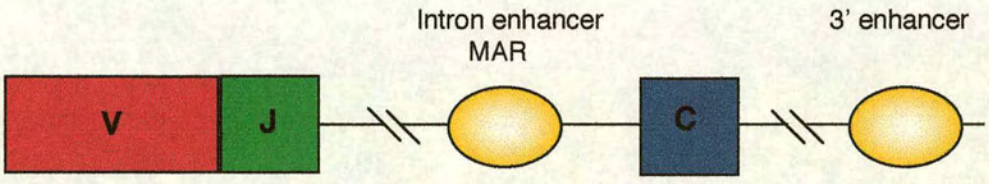
In the search to find out what targets mutation to V regions Sharpe *et al.* (89) investigated the surrounding region of DNA, and found that elements located between 1kb and 9kb 3′ of the Cκ gene were necessary for hypermutation of the V region. They studied the response to Ph-OX in a κ transgene with 9kb 3′ of the C region and found that mutation was still correctly targeted to the V region. However, a shorter transgene with only 1kb

3′ of the C gene was not the target for somatic mutation. The 3′ enhancer has since been located to this downstream region and has been found to be essential for somatic mutation (97)(Figure 1.6).

The κ intron enhancer and the closely linked matrix attachment region (MAR) are also critical for the process (98). It was thought that this could be due to the role of enhancers as activators of transcription. However, removal of the intron enhancer did not affect transcription as determined by RNA quantitation. Thus the intron enhancer might be required for factor recruitment with transcription being activated by the 3′ enhancer instead. This is thought especially likely as the intron enhancer has binding sites for topoisomerase II, which might be involved in creating single stranded nicks in the DNA. These could be required in the mutation process (90).

It therefore appears that both enhancer elements in the kappa locus are required to activate full mutation although there is dispute over which enhancer is more important (92, 95). Enhancers appear to provide specificity, targeting mutation to the V region. Their action could be direct, by recruiting a factor involved in hypermutation, or indirect, by affecting transcription or chromatin structure (95). In the search to find the method of targeting mutation to the V region specifically, the promoter and the V region DNA itself have been deemed unimportant.

Figure 1.6 Location of the immunoglobulin enhancers.



A rearranged murine κ locus, illustrating the location of the intron enhancer , the nearby matrix attachment region and the 3' enhancer

1.9.4 Mechanism of the mutation process

The intrinsic features of somatic mutation along with the results of transgenic analysis have allowed the formulation of several models to explain the process of somatic hypermutation. These models have been changed and updated as more information has been discovered regarding the involvement of cis-acting sequences and other features. Recently models involving DNA mismatch repair enzymes have been prevalent due to research in a number of knockout animals. However, the conclusion is that mice that lack DNA repair enzymes have pleiotropic defects due to elevated mutation levels throughout the genome and that results from these mice are misleading (99, 100).

In the last 2 years significant progress has been made towards the elucidation of the molecular mechanism of somatic hypermutation. And indeed in a very recent study the 'mutator' itself may have been discovered (101). In 2000 the activation induced cytidine deaminase (AID) was found to required for both class switching and somatic hypermutation in mouse B cells (102). This was the first report of a gene that was required for somatic mutation. The requirement for AID was discovered following augmented class switching in a B lymphoma with induced over expression of AID, which is expressed specifically in GC B cells (103).

AID deficient chimaeras were subsequently made and it was observed that mice suffered from hyper IgM syndrome, in the absence of class switching, and also that they did not accumulate mutations in their V region genes (102). AID deficiency has subsequently been reported as a cause for autosomal hyper IgM syndrome in humans, (104). Despite the fact that AID deficient B cells

cannot undergo class switch recombination (CSR) and somatic hypermutation, B cells develop normally and their activation is not impaired as they form GCs and secrete antigen specific IgM. It was proposed that CSR and somatic hypermutation relied at least in part on a common molecular mechanism (102).

The role of AID in somatic hypermutation has been confirmed *in vitro* in two mutating cell lines: BL2 (105) and RAMOS (106). The centroblast like RAMOS cell line, which undergoes somatic hypermutation was found to have levels of mutation correlating with levels of AID mRNA in different clones. By transfecting these cells with AID expressing vectors, mutation rates in low AID expressing clones were increased. Also transfection of hybridomas, which do not normally mutate as they represent a later stage in B cells differentiation, with an AID expressing vector, induced mutation in these cells. AID has also been shown to be required for gene conversion in chickens (107) implicating it in all B cell specific modifications of Ig genes.

The mode of action of AID is not yet fully elucidated. Initially it was proposed to act as an RNA editing enzyme that modified an RNA molecule coding for a protein common to both the CSR and somatic mutation mechanisms (108). AID has homology to the mammalian RNA editing enzyme APOBEC-1, which alters ribo-cytidine to uridine in apolipoprotein B RNA. It also has a cytidine deaminase RNA editing capacity.

More recently a role for AID in DNA cleavage, which has been implicated in CSR and somatic hypermutation, has been examined (109-111). It has however now been reported to function in a post cleavage step as cells lacking AID still accumulate DNA lesions in target sequences for somatic mutation (112). Most recently AID has been proposed to play a direct role in the

causation of mutation of DNA within the immunoglobulin locus itself. AID deaminates dC in DNA to dU; it has been proposed that uracil-DNA deglycosylase then excises these uracils resulting in gaps in the DNA. These are replicated over resulting in the introduction of transversions in chickens. Inhibition of uracil-DNA deglycosylase shifts the pattern of V gene mutation from transversions to transitions, implicating AID in the alteration of DNA (113).

Despite the ongoing investigation into the role of AID in somatic mutation, it now appears that a more directly associated molecular component has been discovered (101). Much research had been performed into a role for error prone polymerises in somatic hypermutation (reviewed in (114)). Although most of the investigations into specific polymerises gave negative results, pol ζ has been implicated in 2 studies as its absence resulted in decreased mutation rates (115, 116).

The recent finding that DNA polymerase iota is required for somatic hypermutation in a Burkitt's lymphoma cell line is very interesting (101). Pol iota (ι) is a member of the Y family of DNA polymerases, which are specialised for copying DNA lesions and have high error rates. Its function was inactivated, in the same fashion as that of AID was in the BL2 line (105), by insertion of neomycin and hygromycin resistance genes in the place of exons 1and 2 or 3 and 4 respectively. The BL2 line can be induced to mutate by crosslinking sIgM in the presence of a T helper cell line. In the pol iota disrupted cell lines this treatment failed to induce mutation, directly implicating it in the mutation mechanism.

These recent results support a model whereby single stranded nicks introduced into the DNA are repaired in a templated but error prone fashion by one or more error prone polymerises. This idea was first proposed by Brenner and Milstein in 1966 (117). Due to the recent progress in finding molecules involved in the mutation process it appears that we may be close to solving one of the most obstinate mysteries remaining in immunology: the molecular mechanism of somatic hypermutation in B cells.

1.9.5 Triggers of the mutation process

Although the effects and characteristics of this high level directed mutation have been investigated, the factors required for induction of mutation *in vivo* are still unclear. Somatic mutation occurs in the TD immune response and it has been shown that LPS stimulation of B cells is insufficient (118). The participation of $CD4^+$ T helper cells is required for full activation of mutation in GCs *in vivo*. To demonstrate this, nude mice, which neither have T cells nor mutate, were reconstituted with different levels of $CD4^+$ T cells and GC cells were isolated by microdissection and their V regions sequenced. Mutations were found to accumulate in GC in proportion to the number of helper T cells present implicating these cells in the activation of mutation in GC (119).

These results have lead the way to experiments performed *in vitro*, which aimed to isolate the specific molecular interactions involved. Kallberg *et al.* (120) discovered that cognate T cell help and crosslinking of sIg were required to sustain mutation *in vitro* of B cells primed *in vivo*. However, as previously shown (118), LPS stimulation or CD40 ligation were shown to be insufficient.

CD40:CD154 interactions while sufficient for class switching and proliferation are insufficient for mutation induction in B cell lines (121, 122). In the human system Denepoux *et al.* (123) demonstrated that crosslinking of a Burkitt lymphoma cell line with Ig and co-culturing with activated cloned T cells induced mutation.

Mutation was first triggered in naive cells *in vitro* in 1997 by Banchereau and co- workers (124). Human tonsillar sIg D^+ $CD23^+$ cells mutated following crosslinking of their membrane Igs in the presence of activated T cell clones however activation through CD40 in the presence of cytokines IL 2, 4 and 10 was insufficient. This demonstrates that secreted T cell factors are insufficient and suggests that the process may be contact dependant. Huang *et al.* (125) subsequently demonstrated mutation initiation in normal human IgD+ B cells in the presence of activated CD4+ T cells in a contact dependant manner.

Recently Zan *et al.* (126) have demonstrated the induction of both mutation and class switching in human IgM+ IgD+ B cell line. Previous cell lines used to investigate mutation have been unable to switch. They found that crosslinking of the sIg was required along with T cell contact through CD40:CD154 and CD80:CD28. This experiment would be even more convincing if it could be repeated with normal naive cells. Experiments described in this thesis and published (127) address this and show that signals via CD40 and the BCR are sufficient to induce mutation in naive cells *in vitro* as long as cell proliferation can be maintained.

All of the above experiments have demonstrated a dependence on signals through the BCR and from the T cell. Neither of these requirements is

surprising as both of these signals are necessary for B cell activation in an immune response.

1.10 Receptor revision

1.10.1 Discovery of receptor revision

Receptor revision is a second process that may play a role in affinity maturation. It involves the secondary rearrangement of variable genes on rearranged Ig loci in the periphery (Figure 1.3), allowing the B cell to alter the specificity of its BCR (Reviewed in (30, 128, 129)). This is a relatively new idea as it was thought that B cells could express only a single receptor, selected during development in the bone marrow.

The term receptor revision refers specifically to secondary V(D)J rearrangements that occur in the periphery, as opposed to receptor editing which refers to the same process if it occurs during development in central lymphoid organs. Until recently secondary V(D)J rearrangement was thought to be restricted to the bone marrow. Han *et al.* and Hikida *et al.* (130, 131) proved otherwise in two papers published side by side in the journal Science. In both papers RAG protein and mRNA were detected in activated B cells from splenic and Peyer's patch GC (and subsequently in draining lymph nodes (132)), suggesting a role for these proteins in V(D)J recombination in the periphery. Functional studies followed with the demonstration of RAG activity in GC B cells. Kelsoe's group (133) detected intermediate products of V(D)J recombination in the B220 ^{lo} subset of GC B cells recovered from immunised C57BL/6 mice; a population which is already destined to die, thus

> 'the improbable genetic experiment of receptor revision is confined to B cells that are destined for programmed cell death'.

These results were concurrent with those of Nussenzweig's group (134), who showed VJ_K and VDJ_H rearrangements in splenic B cells from mice with targeted heavy and light chain alleles ($V_{B1-8}DJ_H2$ and $V_{3.83}J_k2$), cultured *in vitro* with LPS and IL4. Hikida *et al.* (135) also detected excision products reflecting Vλ1 to Jλ1 rearrangement in parallel with RAG expression in mature mouse B cells from the same gene targeted mice, activated *in vitro* with LPS and IL-4. Similar rearrangement was also seen in cells in the draining lymph nodes of immunised mice; *In situ* PCR localised these cells to GCs. Although IL4 has been used to induce RAG expression *in vitro*, it has since been shown that IL7 may be the critical cytokine in the induction of RAG expression in GC *in vivo* as RAG expression is unperturbed in the GC of IL4 deficient mice whereas blocking the IL7 receptor with anti IL7 antibodies greatly decreased RAG expression in GC (136).

In the bone marrow receptor editing is a tolerance mechanism. It is activated in an attempt to rescue cells with auto-reactivity which otherwise would be clonally deleted. The function of receptor revision in the periphery was not quite as clear. As mentioned, Kelsoe's group favoured the idea that receptor revision in GCs was a method of increasing diversity. However, the possibility that it might be involved in peripheral tolerance, possibly of new specificities generated by somatic mutation, had not yet been ruled out. Nemazee's group investigated this question (137) by measuring V(D)J induction as a function of antigen receptor signalling. In their system receptor revision

was measured by the presence of λ expressing B cells in a κ light chain transgenic mouse (3.83 transgenic) - indicating rearrangement of the endogenous light chain. They found that tolerance was unlikely to induce V(D)J recombination in mature splenic B cells as BCR ligation inhibits recombination induced by IL-4 and LPS in splenic B cells *in vitro*. *In vivo*, immunisation of Ig transgenic mice with ligands of varying avidities for the BCR showed that low avidity antigen could induce strong V(D)J recombination whereas non-binding or high affinity antigen could not. If secondary rearrangements in the periphery were in response to self antigens we might expect to see the opposite occurring: V(D)J recombination in the presence of a signal through the BCR. Autoreactive B cells in the periphery are normally removed by deletion when they encounter membrane bound antigen (138).

In human tonsillar B cells Meffre *et al.* (139) showed that mature B cells, expressing non-transgenic antibodies, undergo peripheral V(D)J recombination. The presence of Ig_K signal breaks, intermediates in V(D)J rearrangement, had been determined indicating kappa light chain rearrangement in GC B cells. However, RAG expression and that of terminal deoxynucleotidyl transferase (TdT) – another protein required for efficient antibody assembly - was halted following receptor crosslinking. They propose that B cells, which have already attained high avidity for the prevalent antigen by mutation, would not need to revise the V region of the receptor and hence RAG is switched off. These cells receive a strong signal through their BCRs having bound antigen, which is probably held on the surface of FDC. B cells with receptors that do not have strong affinity for antigen are given another chance, before they die by neglect,

to alter the entire V region and hopefully provide a useful specificity. These cells continue to express RAG.

Although clonal selection implies that cells selected to go into the GC should retain their specificity, new recombination might function to rescue B cells that make deleterious mutations. Or perhaps it could act as an alternative mechanism for producing high affinity antibodies in the GC. Although, as Han (133) says, this is an unlikely genetic experiment, any high affinity recombinants that do arise would be preferentially selected for and clonally expanded. It does appear to be worth the risk.

At this stage the V(D)J recombination machinery had been shown to be active *in vivo* (133,134) and regulated by signalling through BCR *in vitro* in human tonsillar B cells (139). The presence of intermediates of the recombination process indicated kappa light chain rearrangement. In transgenic mice actual V(D)J recombination had been shown to occur in response to low affinity antigen by detecting newly rearranged lambda light chains from the endogenous locus in a κ transgenic system (137).

The above experimental evidence supports the idea that V(D)J recombination in mature B cells exists to increase the diversity of the antibody pool and to increase recognition of foreign antigen and not to prevent recognition of self as it does in the bone marrow. In the bone marrow antigen encounter leads to RAG expression whereas in the GC BCR ligation turns off RAG expression. How can this be the case? Benschop *et al.* have investigated the responses of immature and mature B cells following BCR ligation (140) and found that immature B cell responses require a much smaller increase in intracellular free calcium than does the induction of mature B cells responses.

Thus the cells are intrinsically different in their response to signalling through the BCR. Sandel and Monroe have also demonstrated that the bone marrow microenvironment provides signals that block antigen induced deletion and promote RAG expression (141). In the periphery the absence of these signals allows immature, but not mature B cells, to default to apoptosis as a result of BCR engagement, explaining the different responses of these cells to BCR ligation in the presence of RAG. The cellular constituent of the bone marrow microenvironment was defined subsequently as a novel $Thy1^{dull}$, $DX5^{pos}$ cell that is found in close association with immature B cells *in vivo* (142).

It is difficult to show directly the result of revision, a newly rearranged antibody, without using transgenic systems where the specificity of the BCR is already known. Transgenic systems attract criticism due to the fact that the B cells may be subjected to unnatural pressures to alter their receptor specificities. However, much evidence now exists in transgenic and non transgenic systems that RAG is expressed in GC and that it can cause the secondary rearrangement of V genes in the periphery.

Subsequent investigations into receptor revision in the periphery can be divided into those that focussed on RAG expression, and those that directly examined peripheral rearrangements and their prevalence *in vivo*. The latter can be further subdivided into those which employed transgenic systems and those that selected a more physiological system with a diverse B cell repertoire. Transgenic systems allow easier determination of secondary rearrangements on the basis of loss of expression of the transgenic receptor. Non-transgenic systems require the employment of sequencing of hybridomas generated from

individual B cells to determine the temporal relationship between the onset of somatic mutation and the rearrangement of receptors to localise revision to GC. Both approaches are discussed in subsequent sections, separating investigation into rearrangements at the heavy and light chain loci.

1.10.2 RAG expressing cells in the periphery

The RAG expressing cells in previous studies were found to be localised to GC and to express GL-7, a marker of GC B cells (130, 131). These cells could be mature B cells that re-induce RAG expression during an antigen driven immune response or they might reflect an accumulation of immature cells B cells that continue to re-express RAG having recently emerged from the bone marrow (143). This is supported by the fact that as well as being expressed on GC B cells GL-7 is also a marker of immature B cells. RAG expressing cells were also found to express lambda 5, a component of the pre-BCR (130). A number of groups have investigated the source of RAG expressing cells using mice that co-express RAG and green fluorescent protein (GFP), which can be used as a marker of RAG expression by flow cytometry. Several RAG/GFP transgenic mice have been used in these experiments. One mouse employed has bacterial artificial chromosome (BAC) containing GFP under the control of the RAG2 promoter (144). A second possesses a site directed RAG/GFP fusion gene in which the GFP gene has been inserted into the RAG2 locus (145); a third uses similar technology for the RAG1 locus (146).

The BAC system was employed first by Yu *et al.* (147). They found that cells of an immature phenotype (transitional B cells (148)) continued to express RAG in the spleen, as indicated by GFP expression. They also found that RAG

was not re-induced following immunisation in mature B cells. However, the transitional B cells represented between 5 and 30% of B cells in the spleen making their contribution not insubstantial. RAG expressing cells in the spleen were found to have V(D)J recombination intermediates, detected by PCR, indicating recent gene rearrangement. To determine whether these GFP positive cells could form GC they were adoptively transferred to RAG1 deficient hosts and mice immunised with the hapten NP conjugated to a protein carrier, GFP positive cells (and similarly transferred GFP negative cells) formed GC and made antigen specific IgG1 indicating that they can respond to antigen *in vivo.* Again, however, RAG expression was not re-induced in GFP negative cells and those that were GFP positive lost expression. Re-induction of RAG expression *in vitro* with IL4 and LPS as previously reported (134, 137) also failed.

Yu *et al.* conclude that RAG expression is not re-induced in the periphery following immunisation. These results limit the RAG expressing cells determined in other experiments to a small number of immature cells that may enter the GC before they have turned off RAG expression. These results appear to be contradictory to those of other groups who found re-induction of RAG in splenic cells *in vitro* under the specified conditions. This may be reconciled with the findings of Hertz *et al.* by speculating that low affinity antigen might slow down the maturation of immature cells resulting in the continued expression, rather than the proposed re-induction of RAG (147).

Further investigations into RAG expression in the periphery were performed in the site directed RAG2/GFP mice in which the expression of RAG is proposed to be more physiological. 2 studies found that the immunisation process alone could have an effect on the numbers of transitional B cells in the

spleen (143, 149). This occurs due to a lymphopoietic burst of immature B cells from the bone marrow about 5 days after immunisation. This burst occurs in a non-antigen specific fashion, as the adjuvant alum also has this effect if used on its own, and may account for the perceived re-induction of RAG detected by PCR in previous studies. (143). Gartner *et al* (149) further demonstrated that bone marrow cells were the only one to express GFP in the spleen following adoptive transfer of splenic and bone marrow B cells to RAG 1 deficient hosts. These results were confirmed in the RAG1/GFP system (146).

Despite the strong evidence against RAG re-expression in mature B cells, transitional B cells, present in the spleen, have been shown to continue to express RAG and undergo V(D)J rearrangement (147). These cells have also been shown to form GC when immunised following adoptive transfer to RAG deficient hosts. They may account for the perceived re-induction of RAG reported previously and more importantly may play a role in an antigen specific immune response.

1.10.3 Receptor revision in murine Ig transgenic systems

In murine Ig transgenic systems, receptor revision has been demonstrated in B-1 B cells and in MRL/*lpr* mice. Both B-1 B cells and B cells from MRL/*lpr* mice are prone to autoimmunity, which may be important when interpreting results obtained from these mice. B-1 B cells are a self renewing population of B cells that differ from conventional B cells (B-2 cells) in that they are predisposed to autoantibody production and reside mainly in the peritoneum. Qin *et al.* (150) show that these cells can express RAG1 and RAG2 and undergo secondary V(D)J rearrangement of their Ig heavy and light chain genes. Also, using a

novel genetic mechanism, they show that the rearrangements detected in the periphery occurred *in situ*, rather than having occurred in the bone marrow. The mice used in this experiment express a heavy and light chain site directed transgene (V_{1-8} Hi, 3.83κi). B-1 B cells were found to express BCRs other than those encoded by the transgenes, as detected by loss of binding with an anti-idiotypic (Id) antibody. When idiotype (Id) negative cells were sorted by flow cytometry they were found to express RAG1 and 2 and to contain intermediates in the recombination process. In contrast conventional B-2 B cells most of which are Id positive did not have either RAG expression or V(D)J recombination intermediates.

To determine whether V(D)J recombination was ongoing in these cells rather than having occurred recently in the bone marrow, Ku80 deficient mice were employed. These mice are unable to repair the double stranded breaks introduced by V(D)J recombination and the cells die by apoptosis. B-1 cells were absent in the peritoneal cavity of V_{1-8} Hi 3.83κi $Ku80^{-/-}$ mice. As the B-1 compartment is maintained by self renewal, the absence of these cells in the Ku 80 deficient mice indicates the occurrence of secondary V(D)J rearrangement in an ongoing fashion. RAG levels and the presence of reaction intermediates were also examined in C57BL/6 and autoimmune prone NZB mice both of which were also found to undergo secondary V(D)J rearrangement in B-1 B cells. This is strong evidence for the ongoing rearrangement of V genes in the periphery.

Peripheral light chain rearrangement *in vivo* has also been demonstrated in mice with site directed transgenes specific for single stranded

DNA on the MRL/*lpr* background. These mice have defects in FAS ligand (CD95) resulting in defects in apoptosis (151). In normal mice, B cells that possess transgenic antibodies recognising single stranded DNA are silenced. In the same mice on an MRL/lpr background these cells have been shown to undergo secondary V(D)J rearrangement, possibly in an attempt to avoid autoreactivity. The sequencing of hybridomas generated from the spleens of these mice allowed a comparison of mutation patterns in cells that had undergone secondary rearrangement. This confirmed that the secondary rearrangement had occurred after the onset of somatic hypermutation in the periphery and resulted in the generation of more anti-self specificities (anti double stranded DNA and anti cell nuclei).

In this case it is possible that a normally beneficial mechanism has been turned into a pathogenic process (152) due to the impairment of negative selection in these mice. However, interestingly in this study receptor revision was also found to rescue B cells that had lost the ability to generate a functional antibody due to the inactivation of a light chain by a non-sense mutation introduced by somatic hypermutation, indicating one of the possible benefits of peripheral revision.

Autoreactive B cells should be deleted in the periphery by signals through FAS from FAS ligand expressing T cells. The newly generated autoreactive specificities of these cells would probably not be detected in normal mice, however they are detected here due to the defects in the regulation of apoptosis. The results in autoreactive systems may not give an insight into the normal survival of revised cells but might give us an idea of the frequency of its occurrence in the absence of negative selection. It also allows us to see that the

random generation of new specificities can result in the generation of auto reactive specificities at high level.

The results in autoimmune prone systems concur with a report in a non-autoimmune Ig transgenic mouse model. Magari *et al* investigated the contribution of receptor revision to the generation of high affinity anti p-nitrophenyl acetyl (pNP) antibodies in a mouse with a site directed transgenic heavy chain specific for the hapten NP. Immunisation of the mouse with pNP, which is recognised with low affinity by the transgenic receptor, led to the generation of high affinity antibodies, comprised of heavy chains not encoded by the transgene and newly rearranged lambda light chains. RAG2 mRNA expression and lambda recombination intermediates were detected in pNP immunised mice but not in non immunised mice. These results were reproduced in transfers of sorted lambda negative B cells to RAG1 deficient animals, indicating that the lambda expressing cells that bound pNP with high affinity were produced *de novo* in response to pNP immunisation.

All results in transgenic models appear to support the occurrence of receptor revision in peripheral B cells, however, as always in transgenic systems, there is strong selection for diversification events. Especially in the case of autoimmune mice where there is a defect in negative selection, there is an abnormal bias for this process to be detected. Potential autoreactive specificities will remain due to defects in apoptosis, which removes these cells in normal mice. However, these results do show that mechanistically, receptor revision can occur. The results of studies in non-transgenic systems may prove more informative.

1.10.4 Receptor revision in non-transgenic systems; murine and human.

Due to abnormal pressures for diversification of the B cell repertoire in mice that possess predominantly one BCR, the occurrence of receptor revision may be exaggerated. In systems where the repertoire is already diverse this pressure does not exist, however, receptor revision has still been detected. Firstly in a study of B cells from rheumatoid arthritis (RA) synovial tissue, heavy chain revision was detected at a frequency of around 8% (153). The secondary rearrangements were proposed to have occurred *in situ* due to the detection of RAG expression and the presence of blunt ended double stranded DNA breaks synonymous with the process. Somatic mutation was also shown to have occurred in these B cells. Synovial tissues of some RA patients contain GC – sites of cell proliferation, where this mutation may have occurred. Although receptor revision was found to occur at a high level, RA patients may be prone to dysregulated B cell diversification events leading to an increase in the detection of the event in these patients. It is unclear whether these cells contributed to autoimmune specificities causing disease in this mice.

In a similar study in MRL mice, which also have defects in apoptosis, splenic B cells have been shown to undergo peripheral rearrangement of their heavy chains. This is based on a comparison of the accumulated somatic mutation and secondary rearrangements between neonatal and 4 month old MRL mice with non-MRL mice on the same genetic background (154).

The above studies are in autoimmune models, in normal non-autoimmune humans, one important study detected receptor revision at the heavy chain locus in tonsillar B lymphocytes (155). Following sequencing of hybridomas generated from these individuals, 7 out of 141 sequences

investigated had hybrid V_H segments, indicating the joining of 2 V_H genes in a secondary rearrangement event. These hybrid joins are the only ones detectible by PCR as complete V_H or J_H replacement may be mistaken for germline rearrangements. As a result this may be an underestimation of the actual number of secondary rearrangements in these individuals. Although the investigation of somatic mutation places the secondary rearrangement event temporally after its onset in the GC, RAG expression was not investigated and the possibility that these rearrangements are due to homologous recombination remains.

In contrast to the above results which suggest a high level of receptor revision, in another human study (156) Goossens *et al.* state that receptor revision plays no major role in the shaping of the receptor repertoire after the onset of somatic hypermutation. A similar technique of cloning and sequencing of B cells was employed, however, this time light chain rearrangements were investigated. They estimate the frequency of revised cells at below 3% by comparing single λ cells before and after GC λ formation.

1.10.5 Conclusions

There is now a body of evidence in murine Ig transgenic systems and non-transgenic mouse and human systems, where the repertoire is not restricted, to suggest that receptor revision at both the heavy and light chain loci can occur. It is most easily detectible in autoimmune models where there may be a defect in apoptosis. This means that where receptor revision does lead to the generation

of an autoreactive specificity that it may not be removed in the normal fashion. However revision has also been detected in normal mice and humans.

There exists an apparent contradiction between the information gained from RAG/GFP transgenic mice which failed to show the re-induction of RAG expression in mature cells, with data on V(D)J rearrangement in splenic B cells. In order to reconcile these differences we must assume that either RAG re-expression in the periphery is a very rare event and not readily detectible, or that the cells that express RAG in the periphery are recent immigrants from the bone marrow that are continuing to express it. Although immature RAG/GFP expressing cells have been shown to be able to make an antigen specific immune response, it remains to be shown directly whether these cells are the ones that undergo receptor revision.

There 4 possible consequences for a cell that has undergone receptor revision (128). Due to the difficulties in rearranging a functional receptor, most cells are likely to undergo cell death due to the absence of Ig on the cell surface. Following that, of B cells that do generate a functional receptor, most are likely to have lower affinity for the antigen. If the theory that receptor revision provides useful specificities is correct these should continue to rearrange, as they do not receive a signal through their BCR. Some are likely to generate an autoreactive specificity. In normal individuals these should undergo deletion or anergy, however, these are more likely to remain present in an autoimmune model that has defects in apoptosis, and may explain the detection of revised B cells in these systems. Finally, a rare serendipitous event may result in the generation of a useful specificity that will be selected and clonally expanded. We aimed to investigate this latter possibility, to determine whether receptor

revision in the periphery can contribute to affinity maturation of the immune response.

Aims of this study

1. Triggers of Mutation

The first aim of this study was to investigate signals involved in triggering somatic mutation in B cells. Although $CD4^+$ T cell help and signals through sIg have been demonstrated to be required(119, 120), the specific molecular interactions were still undetermined. We have demonstrated that high levels of mutation can be initiated *in vitro* when naive B cells are cultured with anti-CD40 antibody, anti-Igκ antibody and anti CD38 antibody. Anti-CD40 antibody mimics at least part of the interaction between B cells and T cells. Anti κ antibody provides a signal through sIg mimicking antigen recognition. Anti CD38 antibody is mitogenic for B cells and CD38 deficient mice have also been shown to have defects in antibody responses to TD antigens implicating CD38 in humoral immune responses.

In order to confirm that the above signals were indeed important for mutation initiation *in vivo*, mutation levels were determined in immunised CD40 and CD38 deficient mice. The levels obtained were compared with those of unimmunised and immunised wild type mice.

2. Receptor revision

The aim of the second part of this study is to investigate the hypothesis that receptor revision is involved in affinity maturation. George and Gray (157) have proposed the idea that receptor revision in the GC collaborates with somatic hypermutation in contributing to affinity maturation. It has already been demonstrated that receptor revision can rescue B cells that have lost the ability

to express antibody due to the introduction of a non-sense mutation (151). Therefore It is also possible that secondary V(D)J recombination could allow B cells that have lost the ability to bind antigen by deleterious mutation, to have a second chance at expressing a useful specificity. Receptor revision might allow BCRs to undergo large structural changes, unlike somatic mutation, which only allows small changes to be introduced. The proposed mode of collaboration between the 2 mechanisms is described below.

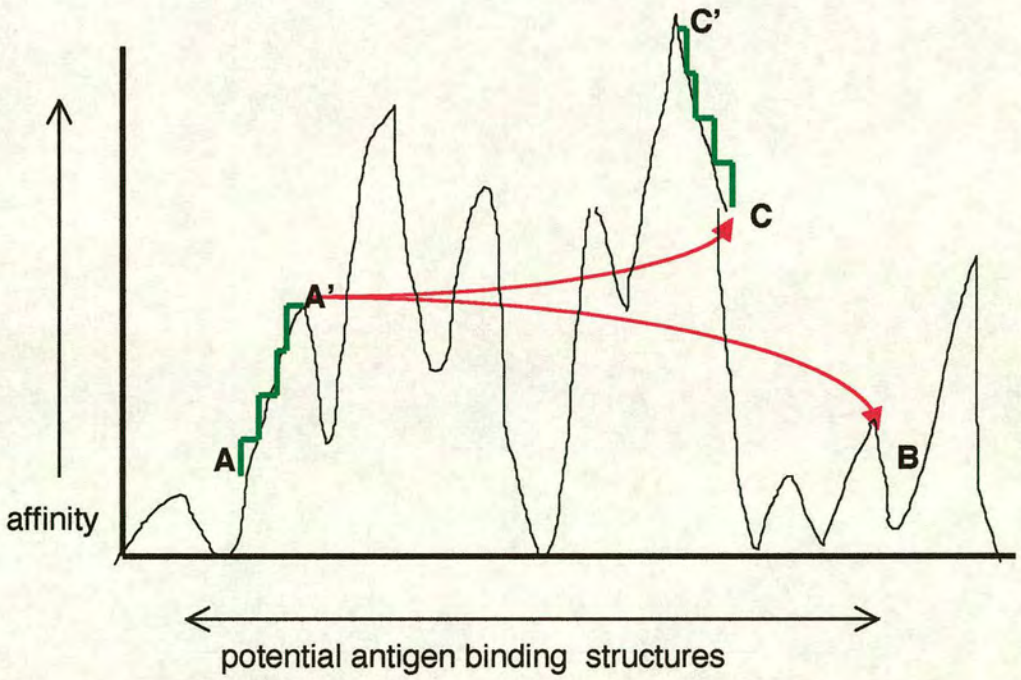
A particular antibody that has been selected in the primary immune response can explore the local area on the affinity landscape by somatic mutation. Large changes in structure could only be caused by receptor revision, which changes the entire V region. This newly formed antibody, which may not have been selected in the primary response is now exposed to the hypermutation machinery, which may allow greater increases in affinity to occur. However, often useless specificities will be created. This idea allows receptor revision and somatic mutation to play complementary roles in affinity maturation.

To explain this idea a hypothetical example is given below. A highly simplified affinity landscape is shown (Figure 1.7). All possible antigen binding sites are shown on the X axis, with the most similar adjacent to each other. On the Y axis the affinity of binding sites for a nominal antigen is shown. A particular antibody that has been selected during the primary response (A), will be altered by point mutation, allowing the immune system to explore the local area around A, by making small alteration in the shape of the antigen binding site (green). Mutations conferring higher affinity are selected for, allowing A to climb the hill until it reached a maximum A'. As mutations conferring lower

affinity are lost due to absence of positive selection in the GC, the antibody cannot undergo changes to increase affinity if they involve a period of loss of affinity. Thus the antibody may be stuck at a local optimum (A'), unable to increase its affinity further.

Receptor revision might allow an antibody to take large leaps through the affinity landscape (red). In most cases this will land the antibody in a locale where the affinity is lower (B). However occasionally the leap will generate antibody on the side of a higher hill (C), which can then use point mutation to climb to the top of that hill (C'). Inhibition of recombinase activity by high affinity interactions ensures that this process is switched off when it is not needed.

Figure 1.7 Affinity landscape demonstrating the potential effect of receptor revision and somatic mutation on receptor affinity



Receptor revision has been shown to occur in peripheral B cells in response to low affinity antigens (137). However it has not been shown directly whether newly gained specificities may contribute to affinity maturation of the response to low affinity antigens. To do this a cohort of transgenic B cells were transferred to adoptive hosts. These hosts were then immunised with antigens recognised with low affinity by the transgenic B cells. The results of experiments performed with two site directed transgenic mice are presented here.

Chapter 2 Somatic hypermutation.

Introduction

Selection of B cells that have undergone mutation of their IgV regions is a cause of affinity maturation. The molecular basis for somatic hypermutation of V genes is still elusive. Neither the mechanism involved nor the molecular triggers have been fully elucidated despite intensive investigation. In this chapter we describe an *in vitro* system to investigate the molecules involved in the initiation of somatic hypermutation. We found that hypermutation could be triggered *in vitro* in mouse B cells by stimulating through three cell surface molecules: CD40, CD38 and Ig (127). In the absence of any one of these stimuli, mutation remains off. The requirement for these signals *in vivo* was investigated in mice deficient in CD40 and CD38. CD40 was found to be obligatory for the induction of mutation, however mice deficient in CD38 had normal levels of somatic mutation, indicating that its role *in vitro* is provided by other signals *in vivo*.

The investigation of somatic mutation in normal mice can be complicated by the fact that many V gene sequences are highly related. This makes it difficult to distinguish between somatically mutated sequences and naturally occurring allelic variation. For this reason a transgenic mouse was employed in this study. ELK transgenic mice possess a Vκ Ox1 transgene, which provides specificity for the hapten Ph-OX, the response to which has been well characterised (158-160). The transgenic construct also carries the 3'kappa enhancer and the intron enhancer, downstream regulatory elements known to be required for the targeting of mutation to V genes (89). The constant region

employed is of rat origin allowing amplification of the transgenic V region only, using a primer specific for this sequence.

The problem of distinguishing V genes in CD40 and CD38 deficient mouse strains, which do not carry immunoglobulin transgenes, was overcome by employing a method first documented by Jolly *et al.* (161). This method takes advantage of the fact that somatic mutations spill over into the region flanking the 3′ side of VDJ_H segments (Figure 1.6). By PCR amplifying the abundantly used J558 family, the VDJ_H 3′ sequence can be compared in different mouse strains. Any one mouse can only carry 2 germline variants of the J558 JH4 flanked sequence used in this analysis; this variation was readily eliminated to allow enumeration of the number of mutations introduced.

The stimuli used in cultures of B cells from ELK mice were designed to mimic signals delivered to B cells during TD responses. Anti Ig was used to mimic antigen-antibody interactions, anti-CD40 to mimic CD154 costimulation from T cells, and Th2 supernatant as it contains cytokines released from activated T cells. We also tested an antibody to CD38, NIMR-5, which has been documented to cause proliferation of murine B cells (162). B cell proliferation is commensurate with the induction of mutation in GC B cells.

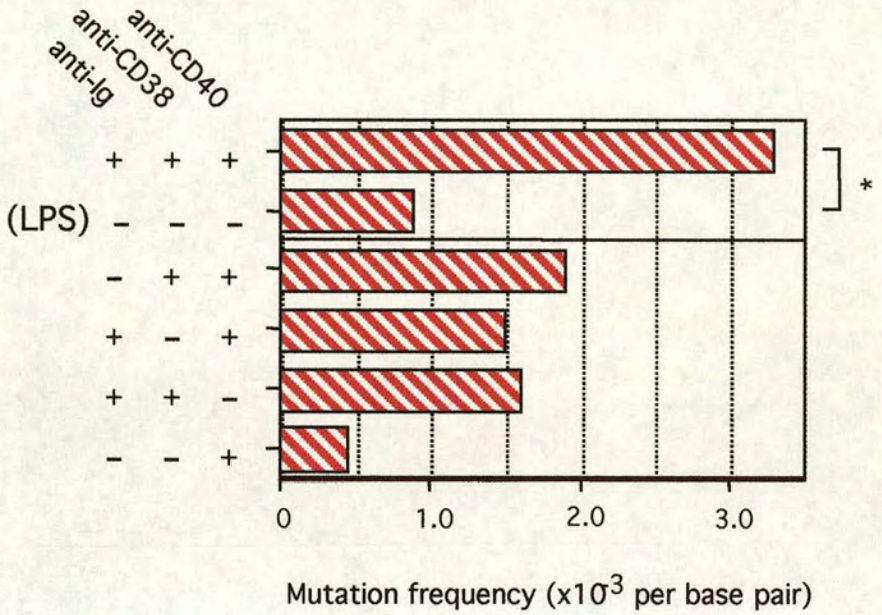
The results presented in the first part of this chapter were attained in collaboration with Siggridur Bergthorsdottir, a previous lab member. Investigation into the proliferative effect of anti-CD38 was performed by myself alone.

Results

2.1 Splenic B cells stimulated in vitro *undergo somatic hypermutation*

B cells from ELK transgenic mice, isolated by CD43 MACS depletion of other cell types, were stimulated *in vitro* with antibodies to various cell surface molecules. We chose molecules that we had reason to believe might be important in the initiation of somatic mutation *in vivo*. $1x10^6$ B cells per well were plated in 24 well tissue culture plates. The following stimuli were added singly or in combination: LPS, anti-CD38 supernatant (provided by Dr Michael Parkhouse, IAH, Pirbright, Surrey), anti-CD40, and anti kappa light chain. Supernatants from alloreactive T cell clones, as a source of cytokines were also used. LPS stimulation alone is known not to induce somatic mutation and was used as a negative control(118). Culture medium and stimuli were replenished after 4 days and B cells maintained in culture for 7 days. After harvesting cells, RNA was prepared, mRNA transcripts from the VκOx1 transgene amplified by RT-PCR, cloned and sequenced. The sequences were compared with the germline VκOx1 sequence and the number of mutations enumerated. Representative results from one experiment are shown in Figure 2.1 and summarised for 4 experiments in Table 2.1 (see appendix for sequence data). Mutation of cultured B cells was only detected in the presence of the three stimuli: anti-CD38, anti-CD40 and anti-kappa. Removal of any one of these stimuli resulted in a statistically significant reduction in the number of mutations introduced.

Figure 2.1 Mutation frequency in the VκOx 1 transgene of cultured transgenic mouse B cells



* P = 0.01

The frequency of mutations obtained in the Vκ Ox transgene following 7-day culture of the B cells with combinations of anti κ (anti Ig), anti-CD40, and anti-CD38. Only the combination of all three stimuli was statistically, significantly different from the LPS control (using Fischer's exact test). This result is representative of three other experiments.

Table 2.I. Frequency of somatic mutations in the V_kOx1 transgene in splenic B cells stimulated *in vitro*.

Stimulus[1]	Number of sequences[2]	Number of mutated sequences	Number of mutations[3]	Frequency of mutations[4]	P[5]
anti-CD38 anti-CD40 anti-Ig	20	9	20	1/290	0.016
anti-CD38 anti-CD40	15	6	6	1/725	0.73
anti-CD38 anti-Ig	14	4	6	1/676	0.52
anti-CD40 anti-Ig	22	9	10	1/627	0.56
anti-CD38 anti-CD40 anti-Ig Th2 supernatant	6	1	1	1/1716	0.75
anti-CD38	8	0	0	<1/2320	0.65
LPS	12	3	3	1/1140	-

[1] Cultures are the same as those from which the sequences in Figure 2.1 were derived.
[2] Number of independent sequences used for analysis.
[3] Total number of mutations in all mutated sequences.
[4] Number of mutations in all sequences divided by the total number of base pairs sequenced.
[5] Probability using Fisher's exact test that the difference in mutation frequency between LPS and triple stimulus is due to chance.

2.2 The pattern of mutations observed is consistent with that of somatic hypermutation induced in vivo.

The mutations incorporated into the VκOx1transgene by the three stimuli were analysed for hallmarks of somatic mutation. The pattern of mutations was scattered throughout the V region, with over half in the FR. *In vivo*, under antigenic selection, accumulation of mutations is normally focussed on the CDRs with fewer in the FR. CDRs are important for specificity as they are the sites that come into contact with antigen, mutations do not normally accumulate in FRs as they are required to maintain antibody structure and function. In our study, the absence of antigenic selection resulted in a broader distribution of mutations and also in equal accumulation of silent and replacement mutations (data not shown).

One of the hallmarks of the mutation process is an increased number of transitions compared with transversions. This is believed to be an intrinsic property of the hypermutation machinery, which is as yet unresolved (90). Transitions are purine (A or G) to purine or pyrimidine (T or C) to pyrimidine mutations whereas transversions are purine to pyrimidine or vice versa. In a previous study transitions have been found to occur with a frequency of 59%, significantly more frequent that the 33% expected by chance (91). In our data set 53% of the mutations are transitions. Even taking into account errors that may be attributable to Taq infidelity, which also favours transitions, the proportion of such mutations is 43%, some 10% greater than the expected frequency (Table 2.2).

Table 2.2. Nature of base substitutions observed in cultured B cells

Mutations[1]	Observed (%)	Expected[2] (%)
Transitions		
Total	56.3	33.3
C→T	18.8	9.3
A→G	25.0	8.0
G→A	6.3	7.4
T→C	6.3	7.0
Transversions		
Total	43.7	66.6
A→T	12.5	8.0
G→T	25.0	7.4
G→C	6.3	7.4

1. Mutations found in the 9 mutated sequences derived from the anti-Ig, anti-CD40, anti-CD38 cultures.
2. Expected number of mutations was calculated by multiplying the frequency of a particular nucleotide (mutation target) in the germ-line sequence by the total number of observed mutations and dividing this by 3 (as mutation to any of the other 3 nucleotides has the same theoretical probability)

2.3 The rate of mutation in cultured B cells is within the normal range.

Calculation of the rate of mutation in this culture revealed that it is in the range previously estimated for somatic hypermutation *in vivo*. The mutation frequency in the triple stimulation culture is 3.4×10^{-3} per base pair. We estimated using the halving of CFSE (5 carboxy-fluorescein diacetate succinimidyl ester) fluorescence that cell in this culture pass through 6 generations (see appendix). Thus the rate of mutation is 5.8×10^{-4} per base pair

per generation. This is 10-fold lower that the maximum quoted rate *in vivo* of $5x10^{-3}$ (163). It has been argued that a rate of $3x10^{-4}$ mutations per base pair per generation is more accurate and indeed most calculated figures are below $1x10^{-4}$ (164, 165).

2.4 Somatic mutation in CD40 and CD38 deficient mice.

To see whether CD40 and CD38 play a role in the induction of hypermutation *in vivo* we immunised CD40 and CD38 deficient mice and analysed the accumulation of mutations in their V genes. 3 mice of each type were immunised intraperitoneally (i.p.) with 100μg of alum–precipitated Ph-OX-chicken serum albumin (CSA), together with 10^9 *Bordetella pertussis* , and boosted 2 weeks later. Spleens were harvested 4 days after boosting and genomic DNA isolated from pooled splenocytes. The J-C intron flanking the 3′ border of VHJ558 genes, one of the most regularly employed gene families, was sequenced following the method of Jolly *et al* (161). This method involves analysis of the region flanking the 3′ side of VDJ_H segments taking advantage of the fact that hypermutation extends into the J-C intron (85).

The advantage of this approach over sequencing of V regions themselves is that the problem of uncertainty over definitively identifying the germline counterpart of the putatively somatically mutated genes is avoided. J_H to C rearrangements were amplified using primers specific for the IgH intronic enhancer and a conserved sequence in FR3 of the V_HJ558 family. The region 3′

of J_H4 is generally heavily mutated in GC B cells (161). For this reason PCR products from J_H4 to C rearrangements were gel purified, cloned and sequenced. The data are summarised in Table 2.3. As expected, in the light of the absence of GC in $CD40^{-/-}$ mice, no somatic mutation was detected (44, 166).

In contrast the J-C intron of B cells from immunised CD38 deficient mice was heavily mutated. This indicates that CD38 is not necessary *in vivo* for the induction or maintenance of somatic mutation. Although *in vitro* , CD38 induced proliferation of B cells, was required for the growth and detection of somatically mutated B cells, it appears that CD38 is not required for the proliferation of B cells in GC.

Table 2.3. Frequency of somatic mutations in the V_HJ558 J/C intron in immunised CD40 and CD38 knockout mice

Mice/Immunisation	Number of sequences[1]	Number of mutated sequences	Number of mutations[2]	Frequency of mutations[3]
Wild-type/ non-immune	13	12	24	1/4660
Wild-type/ immune	11	8	53	1/169
CD40-/- / immune	13	3	5	1/1820
CD38-/- / immune	12	7	38	1/198

1. Number of independent sequences used for analysis.
2. Total number of mutations in all mutated sequences.
3. Number of mutations in all sequences divided by the total number of base pairs sequenced.

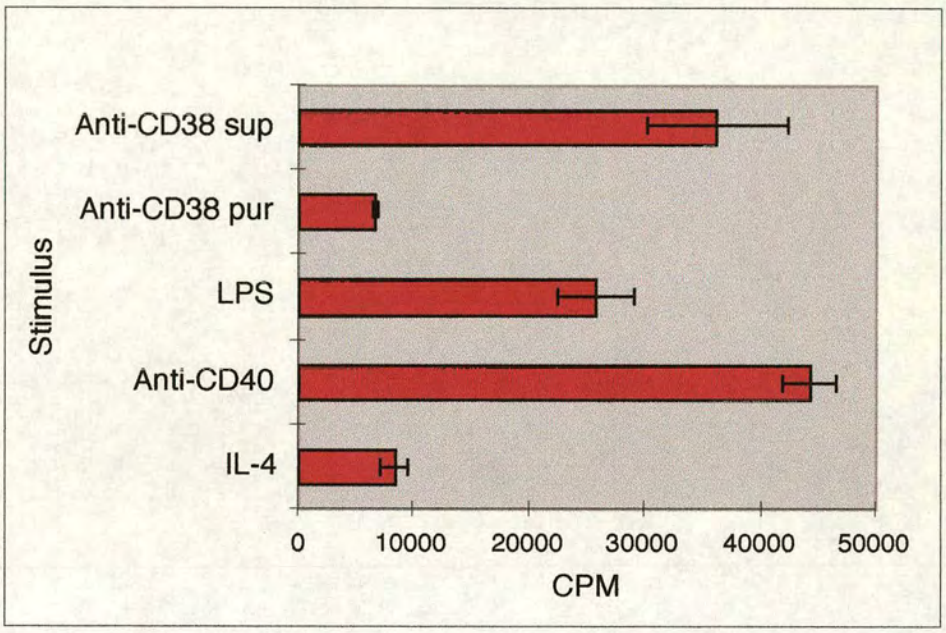
2.5 CD38 supernatant causes proliferation of CD38 deficient splenocytes in culture

The antibody to CD38 used in the experiments described was in the form of supernatant (a gift from Dr M Parkhouse, Surrey) from the NIMR-5 secreting hybridoma. The hybridoma itself was not made available to us. To investigate whether the strong proliferative effect we and others (167) had found it to have on B cells was specific, splenocytes from CD38 deficient mice were stimulated *in vitro* under various conditions. As a comparison anti-CD38 antibody was purified from the supernatant using protein G sepharose. Purified B cells were stimulated *in vitro* and their proliferation measured after 48hr by measuring H^3-thyidine incorporation over 16 hr. Anti-CD38 supernatant was able to induce strong proliferation of CD38 deficient mice, indicating that its effect was not mediated via CD38. In contrast purified anti-CD38 antibody failed to induce proliferation (Figure 2.2).

2.6 CD38 supernatant but not purified anti-CD38 antibody causes division of CFSE labelled cells in vitro.

The proliferative properties of anti-CD38 supernatant were further tested by analysing cell division using CFSE. B cells purified from C57BL/6 mice were purified using MACS CD43 depletion of other cell types. Cells were CFSE labelled and placed into culture at 10^6 cells/ml. Cultures were treated with LPS, CD38 supernatant or purified anti-CD38 antibody. After 6 days cultures were analysed by light microscopy and by FACS. Cultures stimulated in the 3 different ways appeared very different by microscopy.

Figure 2.2 Anti-CD38 supernatant induced proliferation in CD38 deficient mice



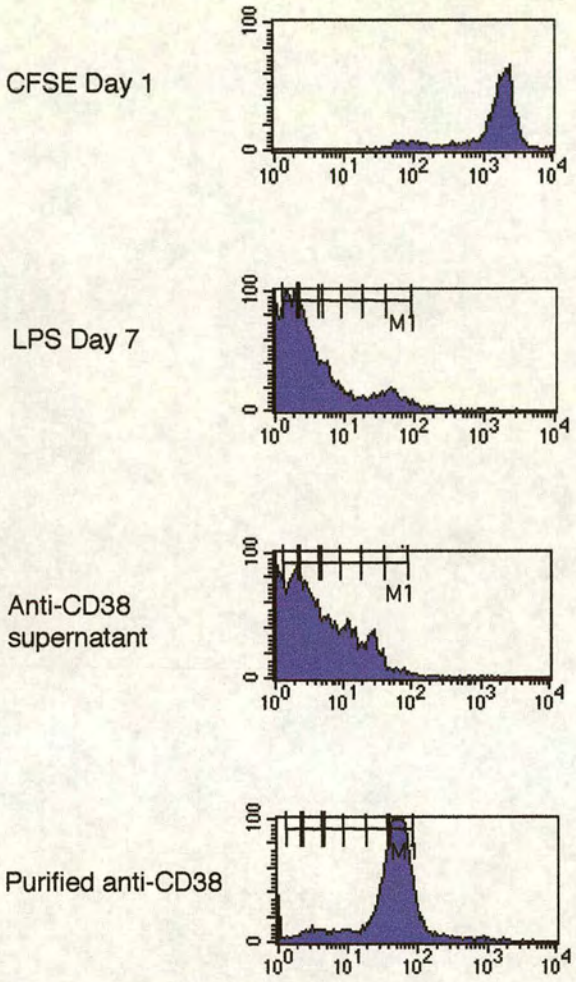
Splenocytes from CD38 deficient mice were stimulated *in vitro* with anti-CD38 supernatant or purified anti- CD38 antibody . LPS and CD40 were used as positive controls for stimulation. H^3 thymidine incorporation over 16hrs was measured after 48 hrs as an indication of cell proliferation. Shown are the means of one experiment performed in triplicate +/- the standard error of the mean. Data shown are representative of two independent experiments.

In cultures stimulated with LPS cells or purified anti CD38 antibody were present singly, however with anti CD38 supernatant cells were present in tight clusters, similar to those induced by CD40 stimulation of B cells. By FACS, firstly purified anti CD38 antibody was tested to ensure that still bound to CD38 expressing lymphocytes. Cell division as measured by reduction in the fluorescent marker CFSE, was induced by both LPS and anti-CD38 supernatant but not by purified anti-CD38 antibody (Figure 2.3), indicating that its effect is not mediated by the antibody but by a component of the supernatant.

2.7 The proliferative effect of CD38 supernatant can be abrogated by the presence of polymyxin B.

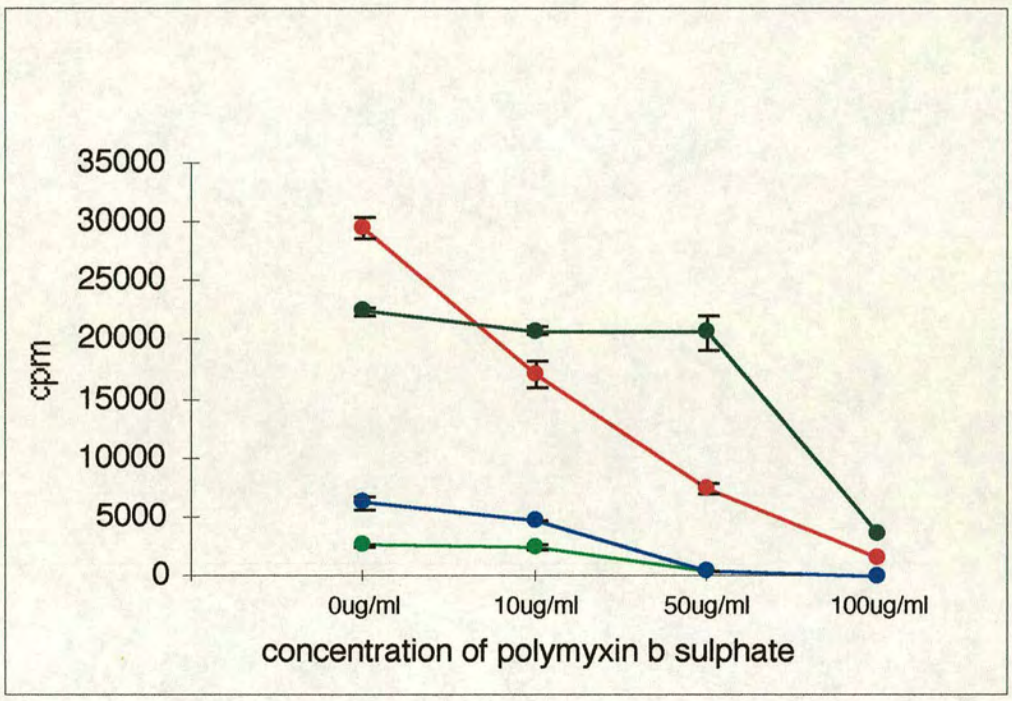
CD38 supernatant but not purified anti CD38 antibody can induce proliferation of B cells in culture. To investigate the contaminating factor in the supernatant that maybe responsible for this proliferation, cells were stimulated with anti-CD40, anti-CD38 supernatant or LPS in the presence of polymyxin B sulphate. Polymyxin B inhibits LPS function by binding it and hence preventing its activation of B cells. After a 48hr stimulation H^3 thymidine incorporation over 16hrs was measured. Increasing concentrations of polymyxin B inhibited anti-CD38 supernatant induced proliferation of B cells (Figure 2.4). In this experiment LPS induced proliferation was low, however, its induction of B cell proliferation is still inhibited in a similar fashion. As a control CD40 induced proliferation was measured this was unaffected by polymyxin B except at a concentration of 100μg/ml where polymyxin B appears to be toxic to cells.

Figure 2.3 CFSE profiles of stimulated B cell cultures



Purified B cells were CFSE labelled and placed in culture with LPS, CD38 supernatant or the purified antibody. 7 days later cultures were harvested and analysed by FACS. Shown from the top are CFSE labelled cells after 24hr in culture, LPS stimulated cells, cells cultured in the presence of CD38 supernatant, or purified anti CD38 antibody. Data shown are from one experiment and are representative of 2 experiments performed.

Figure2.4 Inhibition of CD38 supernatant induced proliferation by polymyxin-B sulphate



Purified B cells were stimulated *in vitro* with anti-CD40 antibody (black), LPS (blue), anti-CD38 supernatant (red) or IL4 (green) in the presence of polymyxin B sulphate at a rangeof concentrations. After 48 hrs H^3 thymidine was added and its incoropration into dividing cellsover 16 hrs measured. Shown are the means of one experiment performed in triplicate+/- the standard error of the mean. Data shown are representative of three independent experiments.

This leads us to conclude that the proliferative effect of CD38 supernatant was caused by bacterial contamination.

Discussion

Background

We have derived an *in vitro* system to define the signals that will initiate somatic mutation in naïve splenic B cells. We find that three signals are required to allow detection of somatic mutation *in vitro*. These signals are anti-CD40, anti-Ig, and anti-CD38. In the absence of any one of these signals mutation remains off. By immunising CD40 deficient mice we have shown that CD40 is required *in vivo*. In contrast CD38 is not required *in vivo* as CD38 deficient mice mutate normally.

These three stimuli were chosen as they mimic signals that B cells may receive in the context of a TD immune response. The GC reaction and somatic hypermutation only occur during responses to TD antigens. This implicates T cells in all of these processes. Although the identity of signals inducing these processes is unknown, CD40 is implicated as CD40 deficient mice fail to class switch and form GCs. Activated T cells express CD154, which provides signals through CD40 on B cells during cognate interaction when the T cell receptor binds antigen bound to MHC class II on B cells. Subsequently CD40 also provides survival signals to GC B cells, which are prone to apoptosis (77) allowing entry into the memory pool (168). Signals via the BCR are critical for the activation of B cells and hence induction of mutation, but also to provide

survival signals during the selection of high affinity B cell mutants with antigen presented on FDCs (169).

It is clear that signals from T cells and through sIg are required for the initiation of somatic mutation *in vivo*. Indeed, it has previously been shown that somatic mutation initiated *in vivo* can be maintained *in vitro* with anti Ig and helper T cells (120). Until now the exact nature of the signals derived from T cells was unknown.

The third stimulus used to initiate mutation in our *in vitro* system was anti-CD38. CD38 is a type II glycoprotein expressed on many cell types including T and B lymphocytes. It has ecto-enzymatic activity, catalysing the conversion of nicotinamide adenine dinucleotide (NAD) to cyclic adenosine diphosphoribose (cADPR)(170). However, it has also been implicated in B cell activation (171). Mice deficient in CD38 have deficiencies in antibody responses to TD protein antigens and it is thought to modulate BCR mediated B cell activation (172). As TD, but not TI antibody responses are diminished in CD38 deficient mice we thought it might play a role in the GC where somatic mutation is known to take place. The role of CD38 in the induction of somatic hypermutation was investigated along with other molecules known to be important in TD antibody responses.

Significance of the results

The difference in the mutation frequency between LPS (1/1140) and the triple stimulus (1/290) cultures is statistically significant. The rate of mutation in the LPS stimulated culture is similar to the Taq error rate (~1/1200) as LPS alone is known not to trigger mutation despite its mitogenic effect on B cells, indicating

that proliferation alone does not induce mutation (118). In the absence of CD38 intermediate levels of mutation were achieved, however these were not statistically significantly different from the LPS rate. This may indicate that CD40 and sIg alone can initiate mutation but that in order for these mutations to be detected at high level that strong cellular proliferation is required; this is provided by anti-CD38 supernatant. One interesting finding in this system was that the addition of Th2 cytokines to the triple stimulus culture inhibits the accumulation of mutations, we think because they drive the differentiation of B cells into plasma cells. This may concur with a study by Razanajaona *et al.* (124) where CD154 transfectants failed to induce mutation in the presence of a mixture of cytokines.

This study is the first report of the induction of mutation in naïve splenic B cells using defined stimuli. In previous investigations into the triggers of mutation, cell lines have been employed (123, 126, 173). Although the information gleaned from these experiments is useful it does not necessarily apply to normal naïve B cells. In one study mutation was initiated at low level in human tonsillar B cells (124) in the presence of T cell clones. The results of this experiment are consistent with results presented here, however the molecular nature of the cellular interaction was not dissected. We have shown that the signal from T cells is through CD40 on B cells.

Pattern of mutations induced in vitro.

The mutation induced in this system using the three stimuli detailed had the hallmarks of somatic mutation induced in passenger transgenes *in vivo* (89). The term passenger transgene is used to describe a transgene that is not expressed

and therefore not affected by antigenic selection. The use of such transgenes has allowed a distinction to be made between the intrinsic properties of the mutation machinery and those imposed by antigenic selection of the most effective mutants. Reported properties for a VkOx1 passenger transgene include a clustering of mutations in the CDRs, accumulation of mutations at hotspots and a transition over transversion preference (88). Of these, the mutations induced in our system had a transition over transversion preference and an accumulation of mutations could be seen in the CDRs, however, we did not note an increase in the serines at codon positions 26,31 or 77 which are intrinsic hotspots for this gene (62). Despite this the rate at which mutations were introduced into the V region DNA was within the range expected *in vivo* ($\sim 5.8 \times 10^{-4}$). This data supports our belief that we have induced somatic mutation as it occurs *in vivo*.

Interpretation of the role of these signals

We have shown that CD40 is required for the induction of mutation *in vivo* . CD40 deficient mice do not form GC as signals through CD40 are required for GC formation. Hence it could be argued that the reason we do not detect somatic hypermutation in CD40 deficient mice is due to the lack of proliferation and antigenic selection in the absence of GC, and not due to the absence of a specific on signal for mutation to occur. Indeed immunised CD40 deficient mice have mutation levels elevated 2.6 fold (1/1820) above those of a non- immune mouse (1/4660). Although this is still 10 fold lower that the level achieved in an immunised mouse (1/200), it may imply that B cells in CD40 deficient mice

maybe able to undergo low levels of somatic hypermutation when stimulated through the BCR.

The role of stimulation through the BCR in the induction of mutation is undisputed. However, our finding that stimulation with anti-CD38 supernatant along with other stimuli can induce high level mutation is novel. The role of CD38 in this culture system is thought not to be as a specific trigger for mutation, but as an inducer of cell division. We believe that CD38 supernatant in combination with other stimuli, drives extensive cell division, allowing the detection of mutated sequences. *In vivo,* GCs are the sites of B cell proliferation, this proliferation is not CD38 dependent as CD38 deficient mice undergo normal somatic mutation of their antibody V regions. However, the strong mitogenic effect of CD38 supernatant used in these experiments is thought to be important.

Subsequent to the publishing of a paper in the Journal of Immunology reporting our findings (127) the nature of the proliferation induced by anti-CD38 supernatant was investigated more closely. We were unable to detect any mitogenic role for purified anti-CD38 antibody, NIMR-5, as was described by Santos-Argumendo *et al.* (167, 174). Also anti-CD38 supernatant was found to be mitogenic for CD38 deficient B cells. This confirms our suggestion that the role of CD38 supernatant in these cultures was simply to induce cell proliferation.

The contaminating factor in these cultures is thought to be of bacterial origin due to the abrogation of proliferation in cells treated with anti CD38 supernatant in the presence of the LPS inhibitor polymyxin B sulphate. It would be interesting to determine whether LPS could replace CD38 supernatant in the

triple stimulus culture. Attempts were made to address using a CD154 transfected fibroblast system, however these experiments were unsuccessful.

Conclusions

This study was the first demonstration of the initiation of hypermutation in splenic B cells using defined stimuli. Antibodies to CD40 and Ig mimic B cell encounter with antigen and interaction with T cells. We believe that anti-CD38 supernatant, which drove B cell proliferation in this system may play a role in mimicking the division of B cells in the GC, however this signal is dispensable *in vivo*. This is also the first formal demonstration that CD40 deficient mice do not undergo somatic hypemutation of V regions.

Chapter 3 Development of an adoptive transfer system to detect receptor revision in QM B cells.

Introduction

The detection of RAG gene expression and activity in GC, has led to speculation that secondary rearrangement of BCRs in the periphery can contribute to repertoire diversity (130, 133, 175). It is now known that in transgenic and non-transgenic mouse models and in humans, B cells can undergo receptor revision (151, 155, 176). This can lead to the generation of autoreactive specificities (150), however it might also lead to the generation of useful specificities that could be recruited to the immune response.

We sought to find out whether B cells, which had undergone secondary V(D)J rearrangement in the periphery might contribute to affinity maturation, which up until now has been solely attributed to somatic hypermutation. To do this a pure population of B cells with the same BCR was required so that any changes in specificity could be attributed to alterations in those cells rather than to expansion of existing specificities. Two mouse systems with targeted insertions of rearranged antibody genes were used as a source of identical B cells to investigate this: the QM mouse (Chapter 3 and 4) and the 3.83 knock-in mouse (177)(Chapter 5).

Quasi-monoclonal (QM) mice were chosen as a source of identical B cells to investigate receptor revision at the heavy chain locus. QM mice carry a targeted heavy chain gene that results in most of its B cells recognising the hapten NP (178). As it has a disrupted kappa chain, it requires the

rearrangement of endogenous lambda light chains in order to make functional antibody. In this mouse secondary rearrangement of the heavy chain generates B cell diversity (179). It is not known what triggers this but it could be low level cross reactivity with a self antigen. There is a strong selective pressure for non-NP specificities and about 25% of QM BCRs no longer express the idiotypic receptor due to rearrangement and selection. This makes the QM mouse immunocompetent and it is capable of responding to a variety of antigens, model and otherwise.

To obtain a pure population of B cells, those cells that have undergone central editing need to be removed by cell sorting. The remaining identical B cells are then transferred to an adoptive host. Experiments are described in this chapter detailing the difficulties encountered and problems solved during the setting up of an adoptive transfer system to investigate affinity maturation of transferred QM cells. Firstly the repertoire of the site directed transgenic, QM mice was investigated.

Results

3.1 The B cell repertoire of the QM mouse includes cells that have undergone central editing.

QM mice express a site directed transgenic BCR, which can be detected on the cell surface using an anti idiotypic antibody. Receptor editing during development results in loss of Id expression in about 25 % of B cells. This is thought to be induced by cross reactivity between the NP specific receptor and self antigens which initiates receptor editing, a tolerance mechanism. However,

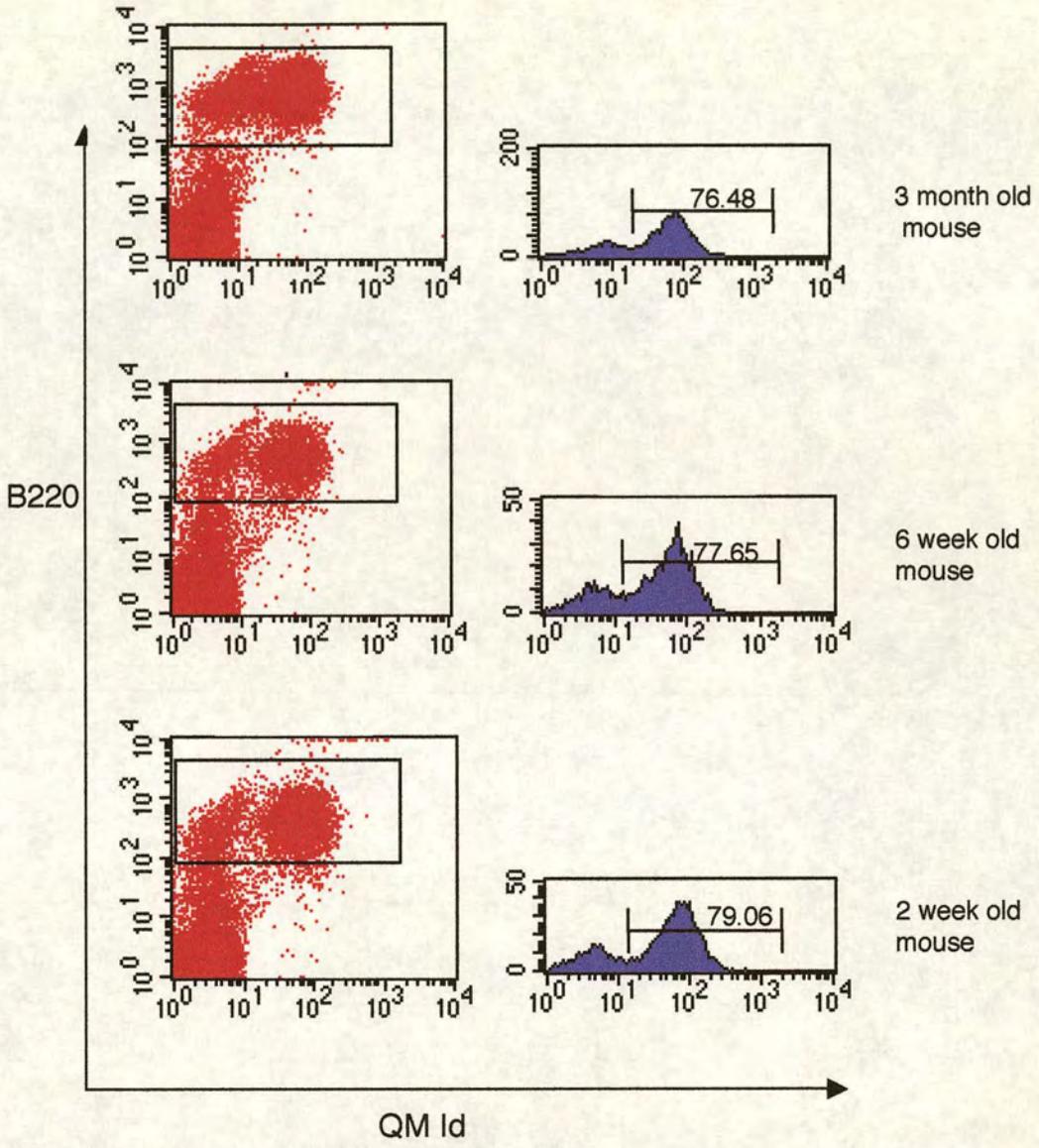
it is also possible that there is cross reactivity with antigens derived from the gut. The latter possibility was suggested to us by Dr Alain Lamarre (Zurich, Switzerland) and would mean that unweaned mice might have fewer of Id negative B cells. To test this, spleen and bone marrow samples from unweaned mice (younger than 3 weeks), 6 week old mice and 3 month old mice were stained with anti QM idiotypic antibody (R2.438.8(178)) and a monoclonal antibody (mAb) against the B cell marker B220.

Mice of all ages examined had similar levels of Id positive B cells (76.5 to 79%) in both spleen (Figure 3.1) and bone marrow (not shown). This indicates that editing in response to ingested antigens is not likely to be a primary generator of Id negative cells as cells were detected at the same level in mice that had not been exposed to ingested antigens. It is, however, still possible that milk antigens or commensal bacteria might be involved in the induction of central editing.

3.2 QM mice can make antibody responses to diverse antigens.

It has previously been reported that QM mice can provide neutralising antibody responses to several viruses (180). This response is presumably provided by Id negative cells, which have edited centrally. To see if centrally edited cells can provide a response to haptens and model antigens, QM mice were immunised i.p. with trinitrophenyl acetyl-ovalbumin (TNP-OVA), dinitrophenyl acetyl (DNP)-OVA, Ph-OX-CSA, recombinant C fragment (RCF) of tetanus toxin and M13 bacteriophage, antigens which were readily available to us. Following a day 10 boost mice were bled on day 14 and serum ELISAs performed.

Figure 3.1 Levels of Id^+ cells in QM mice before and after weaning



To see if levels of Id negative cells were effected by exposure to ingested antigens, spleens were harvested from mice before and after weaning and splenocytes stained with antibodies to B220 and the QM Id. Stained splenocytes are shown on the left. On the right are histograms showing levels of Id^+ cells on the gated B cell population.

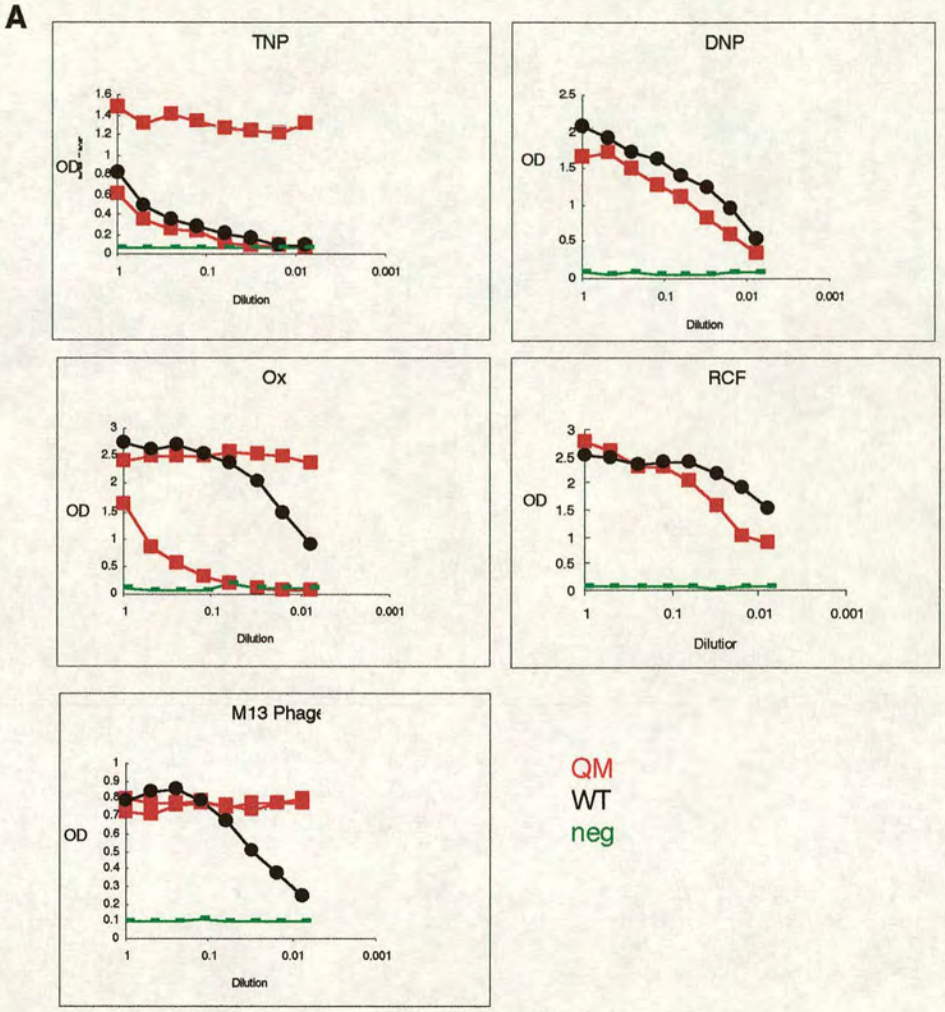
Responses varied greatly between mice probably due to varying responder frequency in the limited QM repertoire, however, for all antigens tested, at least 1 mouse in 2 provided an impressive response similar to, if not greater than, that of a wt mouse (Figure 3.2A).

Further evidence of the response of centrally edited cells was gained visually, by immunising mice with allophycocyanin (APC), a fluorescent antigen. Mice immunised with APC in phosphate buffered saline (PBS) plus 10^9 *Bordetella pertussis* were killed 3 weeks later and cell suspensions generated from spleen samples. Cells were stained with APC to illuminate antigen specific cells and also with a mAb to B220. Mature, $B220^+$ B cells, along with the $B220^-$ compartment reported by Hayakawa *et al* (181) and others can be seen, illustrating the expansion of antigen specific cells in these mice (Figure 3.2B). This also illustrates the need to remove cells of other specificities in order to carryout our investigation into receptor revision.

3.3 The response of centrally edited cells in the QM mouse has normal kinetics

Despite the fact that the QM mice have a limited repertoire (~25% of that of a wt mouse) we had shown they could respond to a range of model antigens. We wanted to test whether the kinetics of this response was similar to a normal mouse. We also investigated the response of the mouse to NP, the antigen recognised by 75% of its B cells. QM and wt mice were immunised i.p. with NP-OVA and DNP-OVA, blood samples were taken at intervals for analysis by serum ELISA.

Figure 3.2 Antigen specific IgG levels in QM mice immunised with a selection of antigens



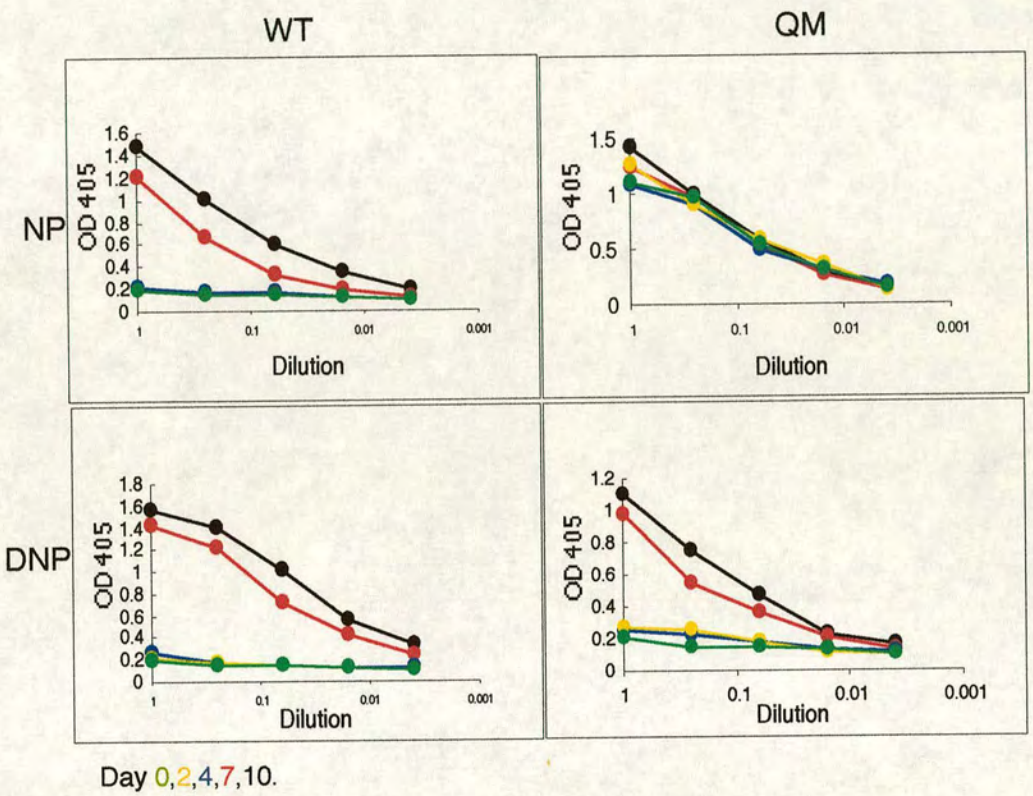
A Immunised mice were boosted on day 10 and blood samples taken on day 14 for serum ELISA. Shown are IgG levels of mice immunised with TNP, DNP, Ox, RCF and M13. QM levels are indicated by red boxes, wt levels are indicated by black circles with negative control levels in green.

B QM mice were immunised and boosted with APC. 2 weeks later spleens were harvested and analysed by flow cytometry for CD19 expression, NP binding and APC binding. Data shown is from one mouse and representative of 3 mice analysed

The DNP response in a QM mouse is similar to that in a wt mouse without any delay in the kinetic of the response (Figure 3.3). This indicates that the limited B cell repertoire in QM mice does not substantially compromise their ability to make an antigen specific antibody response. High levels of NP specific antibody are constitutive in these mice. Unimmunised mice have the same levels as a wt immune mouse and levels do not increase following a normal immunisation protocol. Following investigation of immunisation conditions it was determined that using *Bordetella pertussis* as an adjuvant did raise the response above constitutive levels (Data not shown).

As a further indication of an antigen specific response we counted the numbers of GCs in DNP and NP immunised mice 10 days after immunisation. While those immunised with DNP-OVA had normal GC numbers (22.87+/- 4.8 SD per section n=3) mice immunised with NP either with, or without *Bordetella pertussis* had reduced GC numbers (9.75+/-2.87 SD n=4). This indicates a difficulty in activating QM B cells with NP in a TD fashion within the mouse. Similar findings have been made by the MacLennan lab (Dr Ian MacLennan, Birmingham, personal communication). This is probably due to the high numbers of B cells with the same specificity, as QM cells transferred to other hosts can make GC. These results indicate: 1) That QM B cells do not respond normally upon immunisation with in the QM mouse; 2) that a mouse with a limited repertoire can produce detectible immune responses to diverse antigens. To ascertain whether these specificities are generated by receptor revision and not by expansion of existing cells we need to follow the response of a cohort of identical B cells. This requires the adoptive transfer of QM Id positive B cells to an adoptive host.

Figure 3.3 Kinetics of the response of QM and C57BL/6 mice to NP-OVA and DNP-OVA



Day 0,2,4,7,10.

QM and C57BL/6 mice were immunised with NP-Ova and DNP-Ova. Blood samples were taken and serum IgG ELISAs performed.
WT responses are depicted in the 2 graphs on the left and QM responses on the right. NP responses are depicted in the top 2 graphs with DNP ones below.
In all cases day 0,2,4,7,and 10 responses are represented by green, yellow, blue, red and black respectively

3.4 QM cells can be sorted on the basis of Idiotype expression by flow cytometry

We have shown that QM mice can provide good serum antibody responses to a range of antigens, however this is probably attributable to Id negative cells. Centrally edited cells, undergo V(D)J rearrangement in the bone marrow resulting in loss of Id expression. Receptor revision is the secondary rearrangement of antibody V genes in the periphery and its role, if any is unproven. In order to ascertain whether receptor revision of mature Id positive cells can contribute to affinity maturation of an immune response we needed to obtain a pure Id positive population of B cells, which is not possible in an intact mouse. This was achieved by FACS sorting and performing adoptive transfers of sorted cells. Spleens were harvested from QM mice and cell suspensions made. To increase sorting efficiency B cells were enriched by MACS CD43 depletion. Remaining B cells (~90% pure) were stained with fluorescein isothyocyanate (FITC) conjugated anti-QM Id and anti-B220 conjugated to phycoerythrin (PE), before being sorted on the basis of Id expression using in turn both a MoFlow and FACS Vantage flow cytometer.

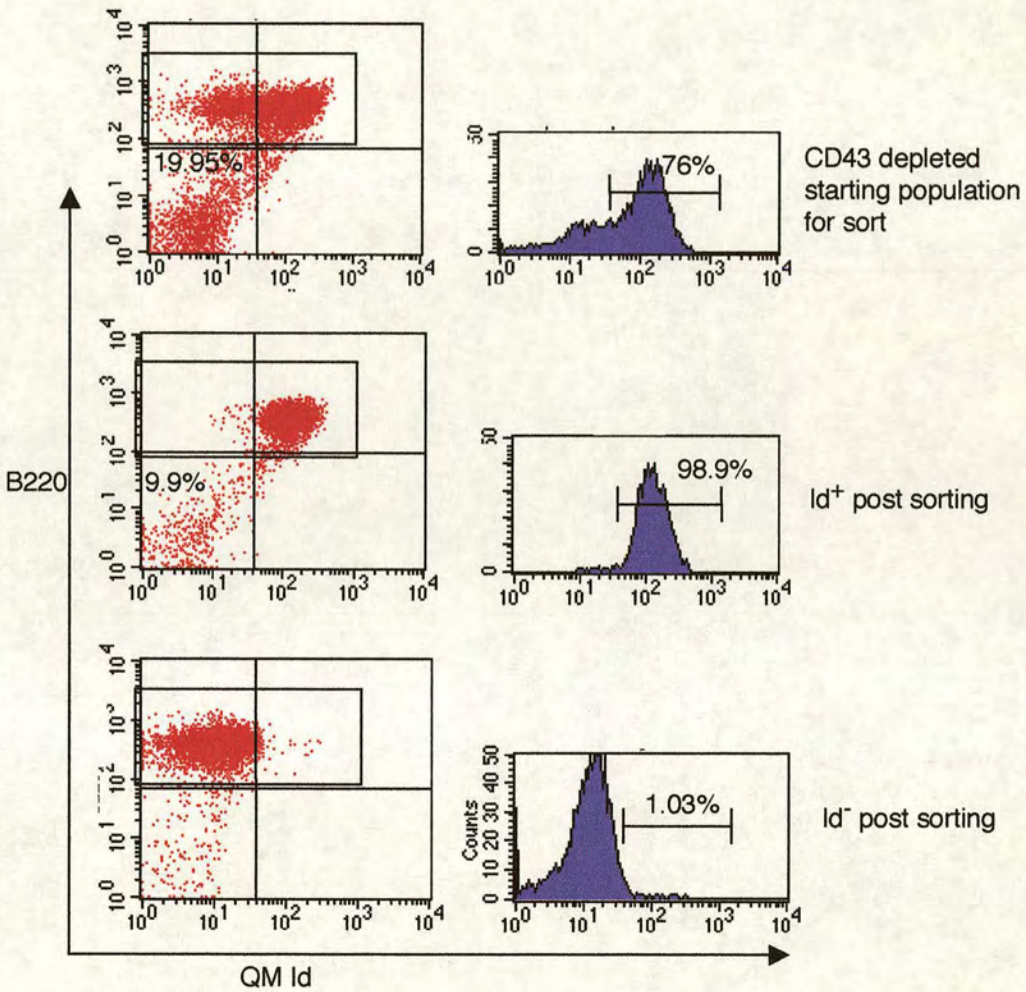
QM mice have small spleens and typically yield only 2 or 3 x 10^7 lymphocytes (normal mice yield around 8x10^7) this is compounded by the fact that only about 25% of these lymphocytes are B cells, probably due to the difficulty in rearranging the lambda light chain. On top of this ~25% of these B cells are Id negative. This means that obtaining good yields following sorting is difficult. Removal of all non-Id positive cells proved problematical. Non-B cells provided the largest contaminating population, however, as these would be

predicted not effect our experiment in any way we did not worry unduly. Id negative B cells were reduced to ~1% of B cells (Figure 3.4). To account for this contamination of our 'pure' B cell population, Id negative cells were also collected and used in control experiments.

3.5 RAG$^{-/-}$ mice are not suitable hosts for adoptively transferred B cells

During the development of an adoptive transfer system for the receipt of a pure population of sorted B cells, RAG$^{-/-}$ mice were selected initially as a potential adoptive host. Numerous groups (143, 149) have employed these mice, primarily as they have no mature lymphocytes. This allows uncomplicated determination of the response of transferred cells. Following preliminary, unsuccessful transfers of C57BL/6 splenocytes to C57BL/6-RAG 2 $^{-/-}$ mice it was decided to irradiate the mice to provide space for transferred cells. Although RAG$^{-/-}$ mice have no mature lymphocytes due to their inability to rearrange B and T cell receptors, early progenitors, developmentally blocked at the early pro B cell stage, reside in the spleen. These cells might compete with transferred splenocytes for space. Two mice in each group were irradiated in 200 rad increments up to 600 rads, 24 hours later $5x10^6$ splenocytes from C57BL/6 mice were injected through the tail vein and mice were immunised i.p. with DNP conjugated to keyhole limpet haemocyanin (KLH). Following a day 10 boost mice were bled and spleens harvested on day 14. Serum antibody responses and transferred cell numbers in the spleen were assessed.

Figure 3.4 Purity of Id positive cells following FACS sorting



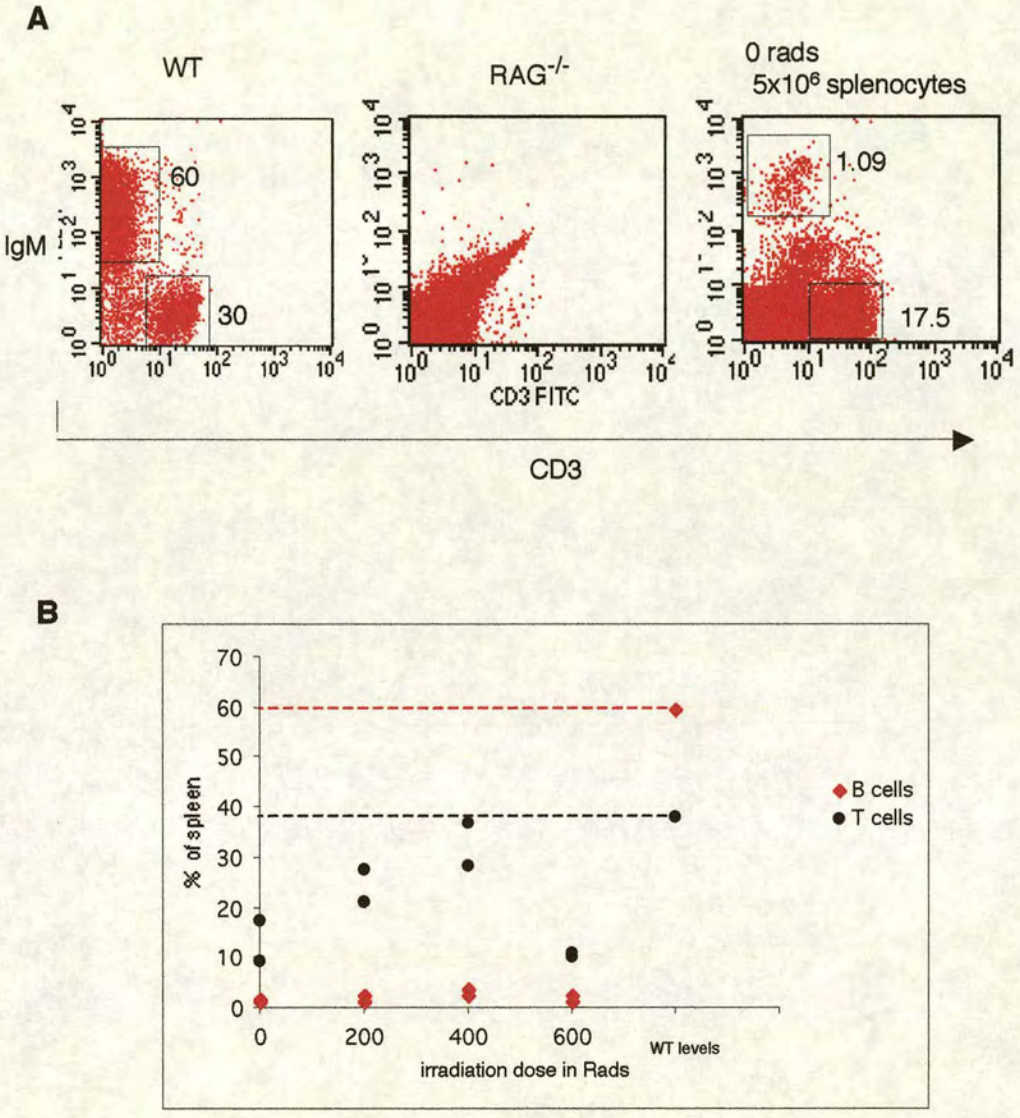
Splenocytes were harvested from QM mice and stained for QM Id and B220 expression. CD43 depletion by MACS enriched for B cells, then Id positive and negative cells were sorted using a FACS Vantage flow cytometer.
On the left whole cell populations are shown, these are then gated on B cells to estimate the proportions of Id positive and negative cells in each sorted population. From the top are shown:- the pre sort population, Id^+ sorted cells and Id^- sorted cells. Data shown is from one sort and is representative of at least 20 sorts performed.

Cell suspensions were stained for T and B cells using mAbs to CD3 and IgM respectively. These markers were selected as they are absent on the immature cells present in $RAG^{-/-}$ mice. T cell numbers approached normal levels (~40% of the spleen) when irradiated at 200 or 400 rads, indicating reconstitution of the spleen with transferred T cells. B cell numbers, however, remained very low (<2% of the spleen) indicating that RAG deficient mice are not a good host for transferred mature B cells (Figure 3.5).

Interestingly, transferred B cells, although they do not fill up the resting B cell compartment, do provide antigen specific serum IgM (but not IgG) antibody responses. This finding was further investigated by staining tissue sections with antibodies to IgM, T24 and with PNA, a lectin that binds to GCs. As intimated by the FACS results, B and T cell areas were absent or paltry, also, there were no GCs. IgM^{+} plasma cells, however, were present in abundance explaining the presence of serum IgM ELISA (Figure 3.6). This phenomenon was subsequently reported and explained by Agenes *et al.* (182).

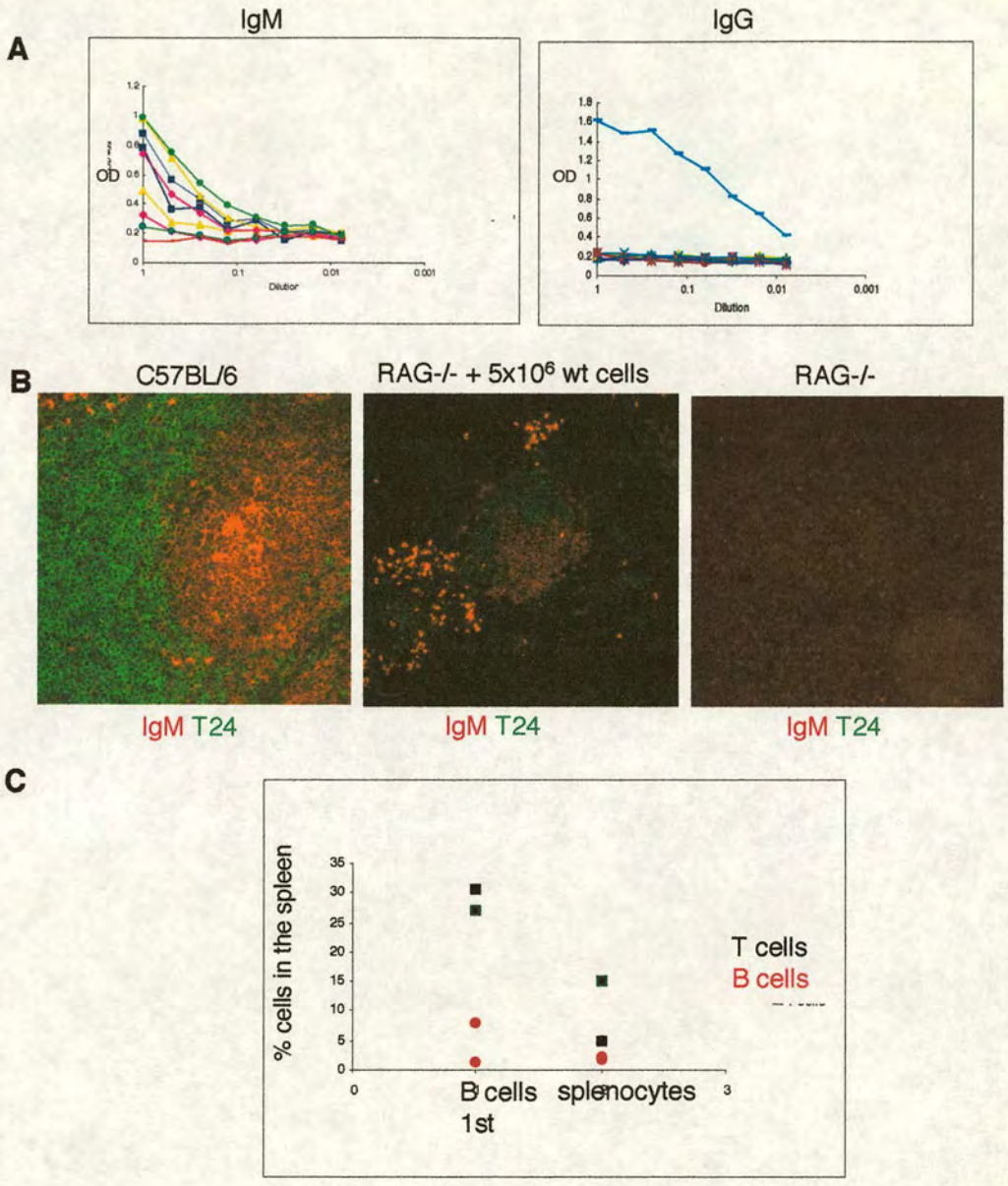
It was suggested to us by Dr Paul Garside (P. Garside, Glasgow, UK, personal communication) that transferring B cells first, followed by T cells 48hrs later might improve B cell reconstitution. To investigate this, mice were given 300rads of irradiation, as this appeared to have given the best results in the experiments described above (Figure 3.5). 2 mice in each group were then given either a) $1x\ 10^7$ B cells and $2.5x10^6$ carrier primed T cells 48 hours later or b) $1x\ 10^7$ splenocytes. Mice were then immunised 24hrs post transfer with NP-OVA. Serum ELISA results similar to previous experiments were obtained, there were good IgM levels but no IgG response to NP (data not shown).

Figure 3.5 Reconstitution of RAG$^{-/-}$ mice with wt splenocytes.



RAG$^{-/-}$ mice were irradiated in 200 rad increments up to 600 rads. 24 hr later 5x10^6 wt splenocytes were transferred and mice immunised with DNP-KLH. Following a day 10 boost, mice were bled and spleens harvested on day 14 for FACS analysis. Splenocytes were stained with anti-CD3 and anti-IgM antibodies to detect T and B cells respectively and compared with a wt and and RAG$^{-/-}$ mouse (**A**).
The percentage of the spleen accounted for by each cell type was counted and is represented in **B**.Black dots indicate T cell percentages in each of two mice per group. B cell percentages are represented by red diamonds.
Dashed lined indicate the normal % of cells in a wt mouse.

Figure 3.6 IgM secretion of resting B cells when transferred to RAG$^{-/-}$ mice



A Serum IgM levels were measured in irradiated mice that received 5x10^6 wt splenocytes. Mice that were unirradiated are depicted in blue, those that received 200rads; yellow; 400rads,pink; 600rads, green and negative control, red. Serum IgG levels: positive control,blue.
B.Spleen sections from (L to R) wt, unirradiated RAG-/- with 5x10^6 splenocytes and RAG-/- stained with anti-Thy-1 FITC (green) and anti- IgM Texas red. B and T cells can be seen, note the IgM $^+$ plasma cells in the middle picture.
C.B and T cell levels in RAG$^{-/-}$ mice that received either B cells first and T cells 48 hr later or splenoctes, presented as percentages of the spleen. B cells are represented as red circles, T cells as black squares.

Reconstitution of the spleen was measured by FACS analysis (Figure 3.6). In one of the two mice in which B cells were transferred first there appeared to be an increase in splenic B cell levels, however full reconstitution was not achieved. We decided that in order to have the correct environment for GC formation and affinity maturation, normal B and T cell areas were required, and so the $RAG^{-/-}$ mice were not suitable for this experiment.

3.6 Analysis of $CD40^{-/-}$ mice as a potential adoptive host.

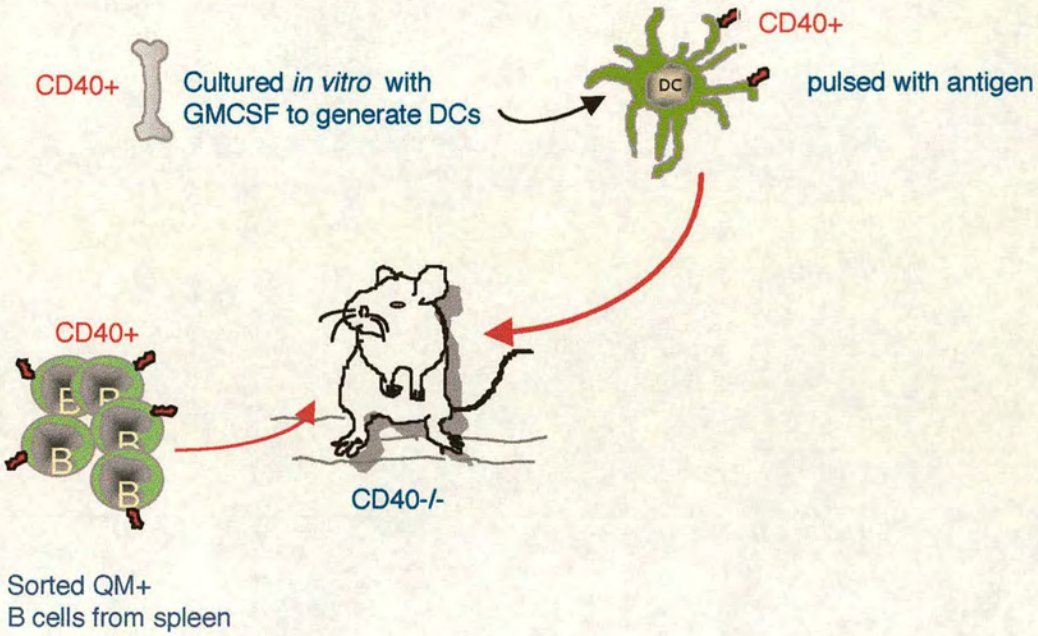
The experiments we had performed with $RAG^{-/-}$ mice demonstrated the need to find a suitable adoptive host to receive QM Id^{+} cells. $RAG^{-/-}$ mice appeared to have the advantage of having no complicating host cells, this however, limits their use as a physiologically relevant model in the failure to reconstitute them successfully. Other potential adoptive hosts come with the complication that they carry their own endogenous lymphocytes; hence we would need a way to distinguish the response of host cells from that of the transferred cells. Other groups have used congenic strains with an allotype marked heavy chain (33) mice with cell surface or clonotypic markers (183), or haemopoietic markers (182). We decided to use $CD40^{-/-}$ mice as they possessed several advantages over other potential hosts and they were also readily available to us.
Transferred cells can be detected by FACS on the basis of expression of 2 cell surface markers – the QM Id but also, in the case of loss of expression of this, they express CD40 which is lacking in the knockout host.

A second advantage of this system is that $CD40^{-/-}$ mice are unable to make class switched antibody responses. This is due to a) the absence of CD40 on DCs, which is required for the activation of T cells and b) the absence of CD40 on B cells themselves, which needs to be ligated by T cells during cognate interaction to induce class switching in response to TD antigens. This means that adoptively transferred $CD40^{+}$ B cells will be the only cells in the mouse able to produce IgG, thus allowing us to monitor their response by isotype specific ELISA. However, to circumvent the lack of CD40 on DCs and allow priming of helper T cells these mice must be supplemented with $CD40^{+}$ DC (Figure 3.7).

$CD40^{-/-}$ mice which have received wt B cells fail to make a class switched response, however, if splenocytes are transferred the $CD40^{+}$ DCs in the spleen enable the $CD40^{+}$ B cells to respond (Figure 3.8). As QM cells need to be sorted, DCs need to be added separately. Immunisation of $CD40^{-/-}$ mice with DCs while activating the T cells and causing their migration to B cell follicles, does not result in class switching of $CD40^{-/-}$ B cells (S. Fillatreau and D. Gray in press and Figure 3.8) confirming that IgG can come from the transferred $CD40^{+}$ B cells alone.

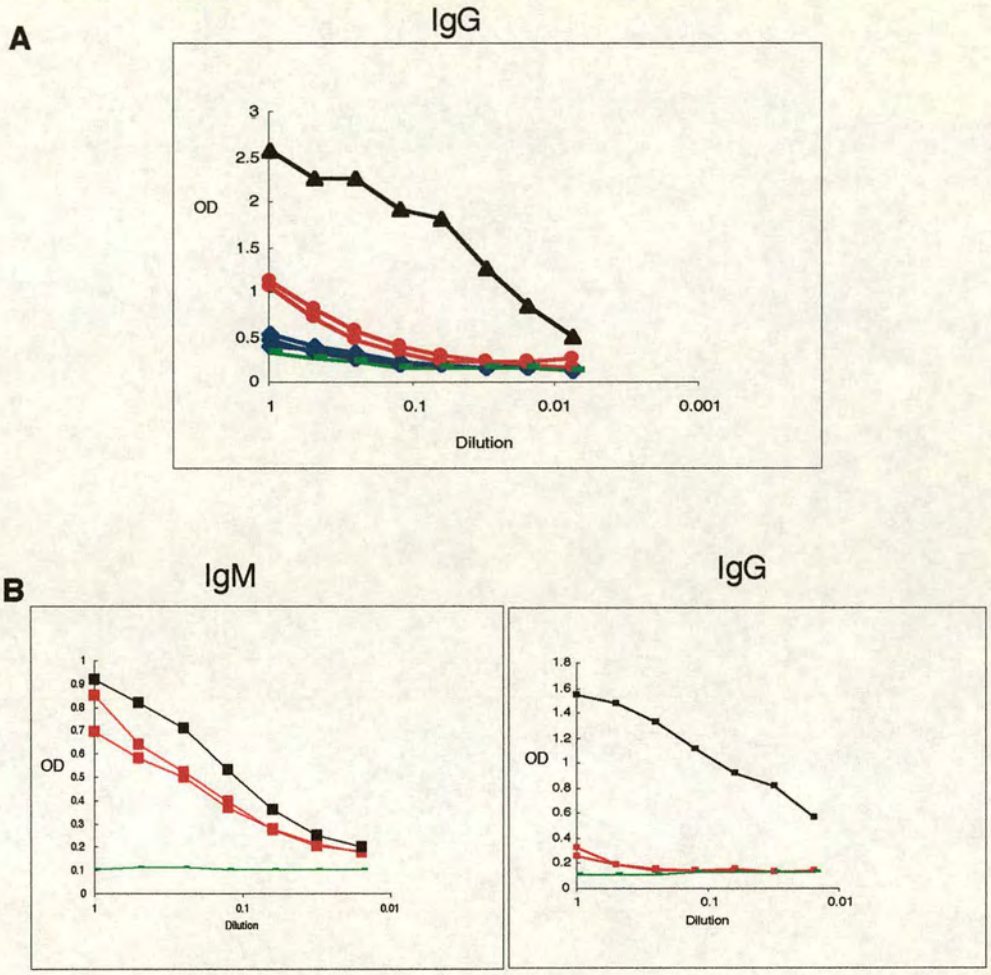
Bone marrow derived DCs were generated *in vitro* in a 7 day culture with GM-CSF. These DC were pulsed with NP-OVA at 100μg/ml for 1 hour at 37^{0}C before being harvested.

Figure 3.7 Immunisation of $CD40^{-/-}$ mice with pulsed $CD40^{+}$ DCs allows activation of transferred $CD40^{+}$ B cells.



A schematic diagram illustrating the transfer protocol to $CD40^{-/-}$ mice. Bone marrow derived dendritic cells, cultured in vitro were pulsed with antigen and used to immunise $CD40^{-/-}$ mice. Sorted QM Id^{+} cells were simultaneously transferred.

Figure 3.8 Antibody responses in CD40$^{-/-}$ mice that received transferred splenocytes or B cells alone.



A. CD40$^{-/-}$ mice received either 1.5x10^{7}B cells (blue) or 5x10^{7}splenocytes (red) and were immunised with DNP-KLH 1 week later. Blood samples were taken on day 10 for serum IgG ELISA. The positive and negative controls are indicated by a black and green line respectively.

B. CD40$^{-/-}$ mice that received 4x10^{6} QM B cells were immunised with NP-OVA pulsed DC and blood samples taken on day 14. IgM levels are shown on the graph on the left and IgG levels on the right, in both graphs red lines indicate experimental mice with black and green lines for positive and negative controls respectively.

$2x10^6$ DC derived from C57BL/6 mice and $4x10^6$ QM B cells were mixed and injected i.v. to $CD40^{-/-}$ mice which had been backcrossed for 7 generations onto the C57BL/6 background. Although IgM was produced by host B cells, indicating the success of the immunisation by DC, IgG was not produced by co transferred QM B cells (Figure 3.8). This experiment was repeated 3 times, altering the quantity of antigen and length of pulse with similar results. GC were not formed but most tellingly transferred cells could not be detected by FACS. At the time this result was assumed to be related to sub-optimal activation of transferred cells, however, it later became clear that it was graft rejection due to incomplete backcrossing of the QM mice.

3.7 Development of a chimaeric mouse with CD40 on DC but not B cells.

Assuming that our failure to detect a response from transferred B cells in $CD40^{-/-}$ mice was caused by DC and B cells not interacting at the correct location, it was decided to make a mouse in which $CD40^{+}$ DC and $CD40^{-/-}$ B cells coexisted. A bone marrow chimaera system was under development in the lab to allow complete substitution of B cell and DC compartments. This work is presented as a collaboration with many lab members, most notably Simon Fillatreau.

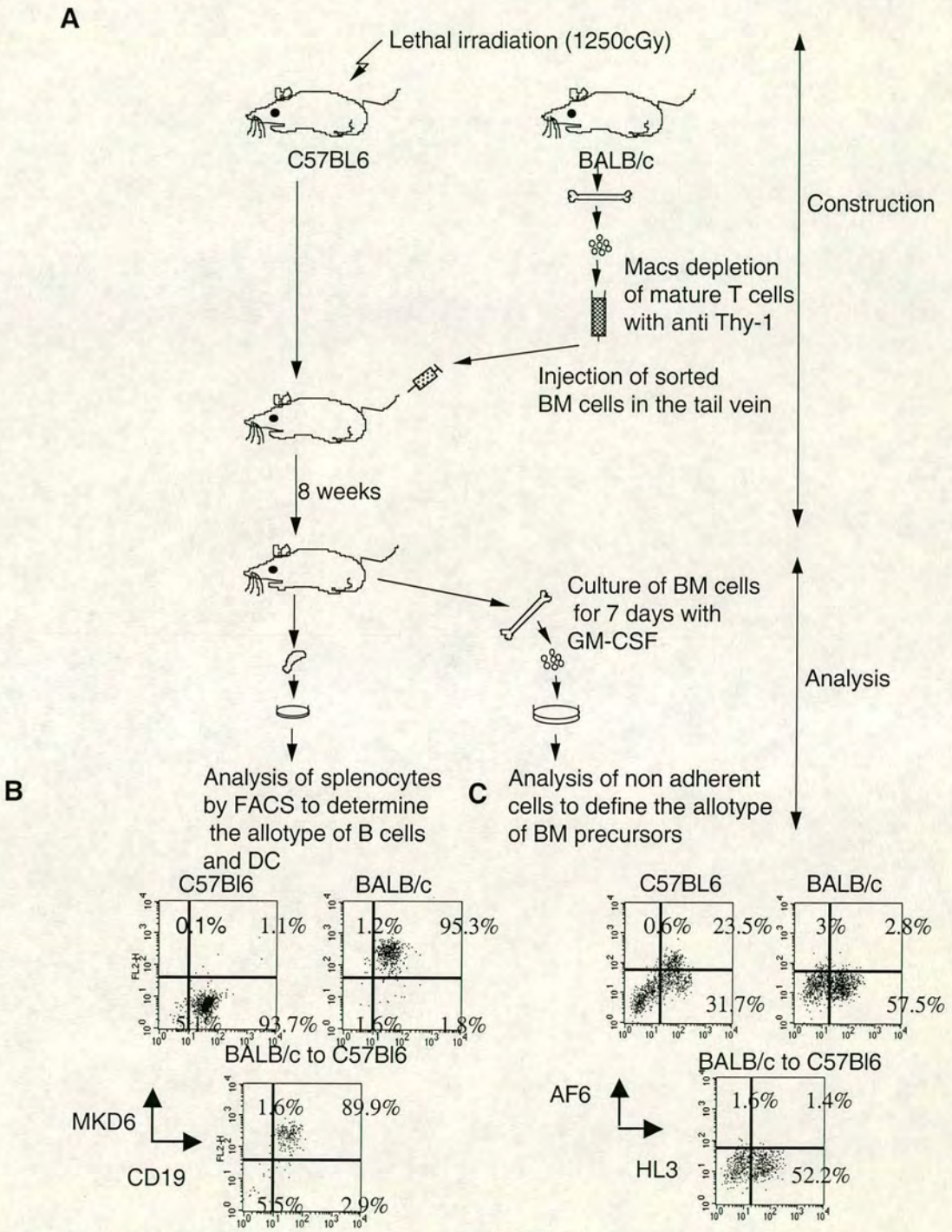
During the development of this system allogeneic transfers were made so that reconstituted cells could be distinguished from those of the host. This allows us to be sure that the chimaera is comprised only of transferred cells.

The procedure is illustrated in Figure 3.9, briefly, host C57BL/6 mice were lethally irradiated. 24hrs later they were reconstituted with $5x10^6$ bone marrow cells, depleted of mature T cells, from BALB/c mice. Mice were left 8 weeks to reconstitute and then analysed. Splenocytes were analysed by FACS to determine the MHC class II haplotype of B cells and DC. BM cells were also cultured and non adherent cells analysed by FACS to define the haplotype of bome marrow DC precursors. Representative data is shown for splenic B cells and bone marrow precursors using either mAbs AF6 (anti IA^b) or MKD6 (anti IA^d) although both populations (and also splenic DCs) were stained with the two antibodies. The splenic B cells, DC and bone marrow DC precursors in the reconstituted chimaeras were derived only from cells in the transferred bone marrow, without any contribution from host cells, confirming complete removal of the host immune system by irradiation.

The chimaera used for the adoptive transfer of QM B cells has CD40 deficient B cells and CD40 sufficient DC. To generate this chimaera C57BL/6 mice were irradiated and reconstituted with a mixture of μMT and $CD40^{-/-}$ bone marrow (these are referred to as μMT/$CD40^{-/-}$ chimaeras). μMT bone marrow, lacking B cells provides T cells and $CD40^+$ DC, $CD40^{-/-}$ bone marrow provides the B cell compartment.

To confirm the absence of $CD40^+$ B cells from the host, and hence validate our model, we stimulated B cells *in vitro* with anti-CD40 antibody. Stimulation through CD40 causes B cell proliferation and would amplify any contaminating host cells.

Figure 3.9 Development of a bone marrow chimera system allowing complete substitution of B cell and dendritic cell compartments.

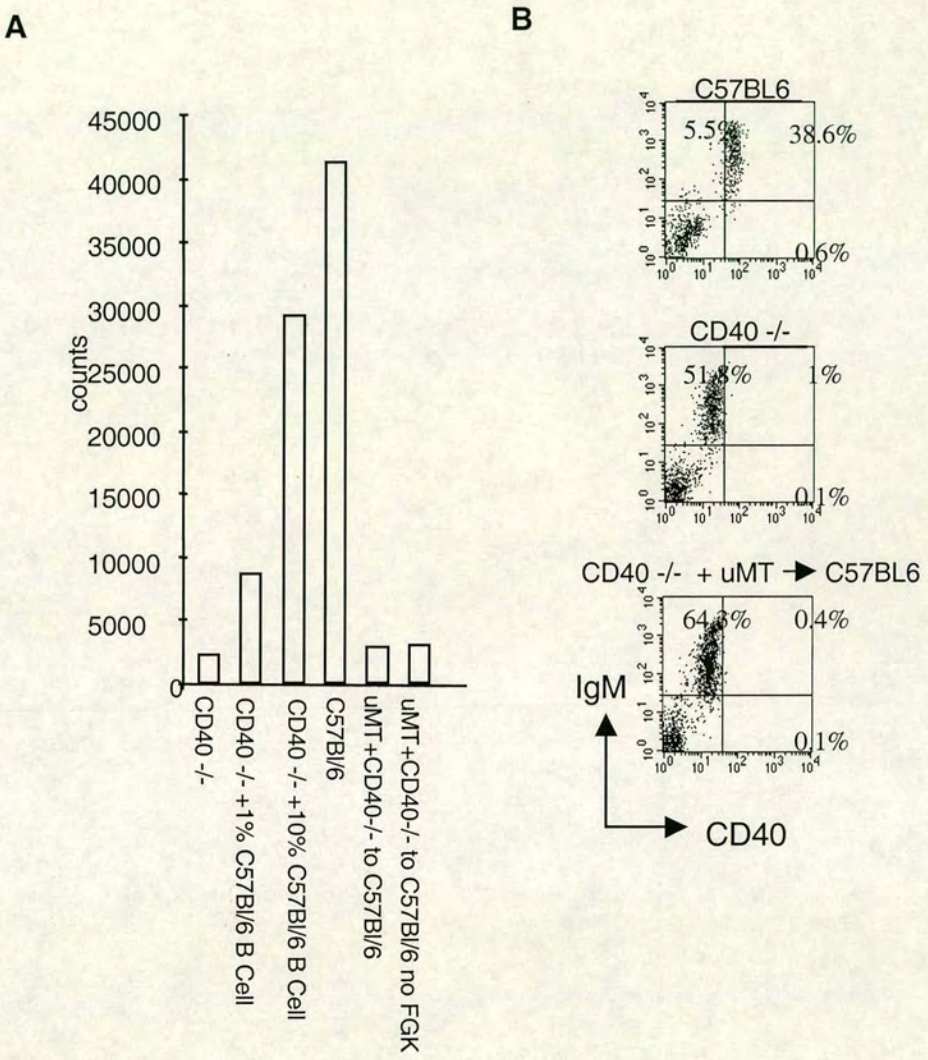


A.Schematic diagram of the development of the chimaera system.
B. Analysis of splenic B cells with anti IA^d antibody MKD6 and CD19, showing staining patterns on a C57BL/6 mouse, a BALB//c and a BALB/c to C57BL/6 reconstituted chimaera.
C Analysis of non adherent bone marrow precursors with anti anti IA^b AB AF6 and anti CD11c (HL3), showing staining patterns on a C57BL/6 mouse an BALB/c mouse and reconstituted chimaera.

Spleens were taken from $CD40^{-/-}$, C57BL/6 mice and reconstituted chimeras. As a control for the sensitivity of the assay $CD40^{-/-}$ cells were also spiked with either 10% or 1% $CD40^{+}$ B cells. Cells were stimulated for 48hrs at which time proliferation was measured by H^3 thymidine incorporation. Resulting counts are shown in Figure 3.10 alongside FACS data confirming absence of detectable CD40 on B cells in reconstituted chimaeras. Control cultures demonstrate that $CD40^{+}$ B cells can be detected even if they represent only 1% of the cells in the assay. This gives us confidence in stating that reconstituted chimeras contain no B cell or DC contribution from the host, and that we have achieved complete substitution of the B cell and DC compartments.

It was determined that reconstitution with 20% $CD40^{-/-}$ bone marrow and 80% μMT bone marrow gave the most physiological levels of all cell types involved. This was determined by histological analysis of spleen sections. T cell priming was also assessed by re-stimulating T cells *in vitro* and assaying proliferation and cytokine production. We aimed to maximise levels of DC from the μMT host, while still allowing reconstitution of the B cell compartment within a 2 month period. 10% $CD40^{-/-}$ bone marrow and 90% μMT bone marrow resulted in unreliable reconstitution of the B cell compartment.

Figure 3.10 Analysis of chimaeras reconstituted with μMT and $CD40^{-/-}$ bone marrow



C57BL/6 hosts were irradiated and reconstituited with 80%μMT bone marrow and 20%$CD40^{-/-}$ bone marrow.

A To confirm absence of $CD40^{+}$ B cells, spleens were harvested from chimaeras 8 weeks after reconstitution and stimulated with anti CD40 antibody (FGK-45). Proliferation was measured by H^3 thymidine incorporation after 48 hrs and plotted (LtoR) $CD40^{-/-}$,$CD40^{-/-}$contaminated with 1% C57BL/6, $CD40^{-/-}$contaminated with 10% C57BL/6, C57BL/6, reconstituted chimaera, chimaera with no FGK.

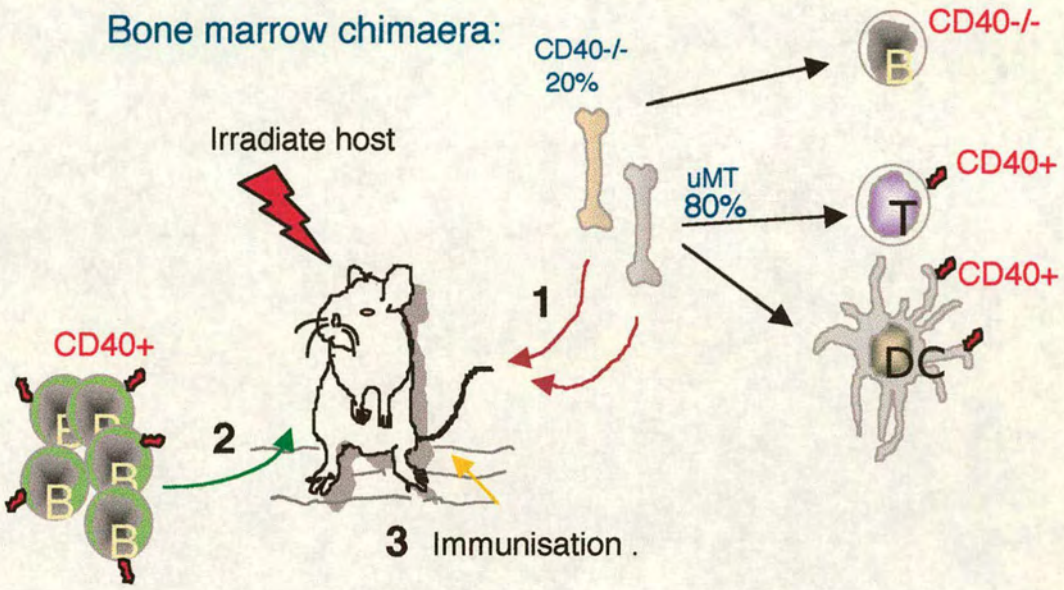
B. FACS staining of reconstituted chimaeras, compared with C57BL/6 and $CD40^{-/-}$ mouse.

3.8 Mixed bone marrow chimaeras make good adoptive hosts for transferred CD40^{+} B cells

We planned to use μMT/CD40$^{-/-}$ chimaeras as adoptive hosts for transferred QM B cells but first, to validate the system, C57BL/6 B cells were transferred. Mice immunised with 2 model antigens, NP-OVA (not shown) and DNP-KLH to confirm that transferred B cells could make an antigen specific antibody response (Figure 3.11). Blood samples were taken at 7 day intervals and assayed for total IgG and IgG1 by serum ELISA (Figure 3.12). Mice were given either $1x10^{6}$ (not shown), $1x10^{7}$ or $5x10^{7}$ B cells. The response was greater and faster in those which received more cells, presumably due to increased responder frequency, however, all mice made some antigen specific antibody response.

DNP-KLH immunised mice were sacrificed on day 16 and splenocytes stained for CD40^{+} B cells. CD40^{+} B cells can be easily distinguished from the CD40$^{-/-}$ B cells of the host by FACS. The mice with increased numbers of CD40^{+} cells proved to be those that had made the greater antibody response, illustrating clonal expansion in these animals. Sections were also cut and stained for GC. GCs were formed indicating that transferred cells can make a TD immune response. In contrast chimaeras that did not receive transferred cells had no GC and no IgG. These results indicate that transferred cells can make a TD response, produce IgG distinguishable from host produced IgM and make GC. Grafts of CD40^{+} B cells also proved to be very stable, in mice tested grafts were still present after 3 months.

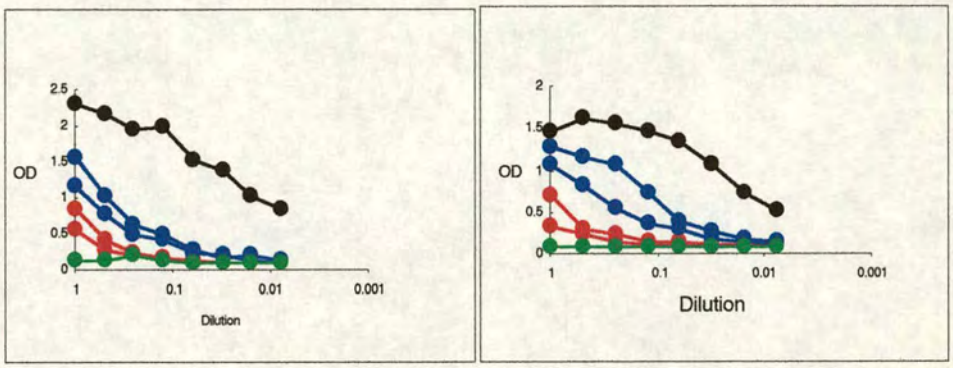
Figure 3.11 Chimaera strategy - a mouse with CD40 on DCs but not B cells allows detection of the response of transferred $CD40^+$ B cells.



A schematic diagram illustrating the construction of μMT/ CD40 chimaeras and their subsequent transfer with $CD40^+$ B cells and immunisation.

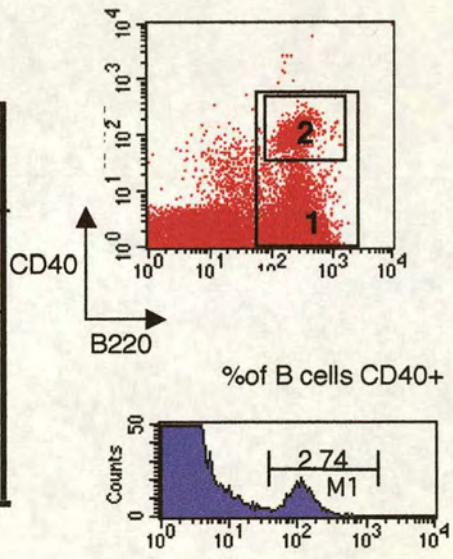
Figure 3.12 Response of CD40^{+} B cells when transferred to chimaeras

A



B

Mouse	%CD40+ B cells in the spleen
1. Negative control chimera	0.21
2+3. 5x10^{7} wt B cells	2.74 7.60
4+5 1x10^{7} wt B cells	2.70 3.13



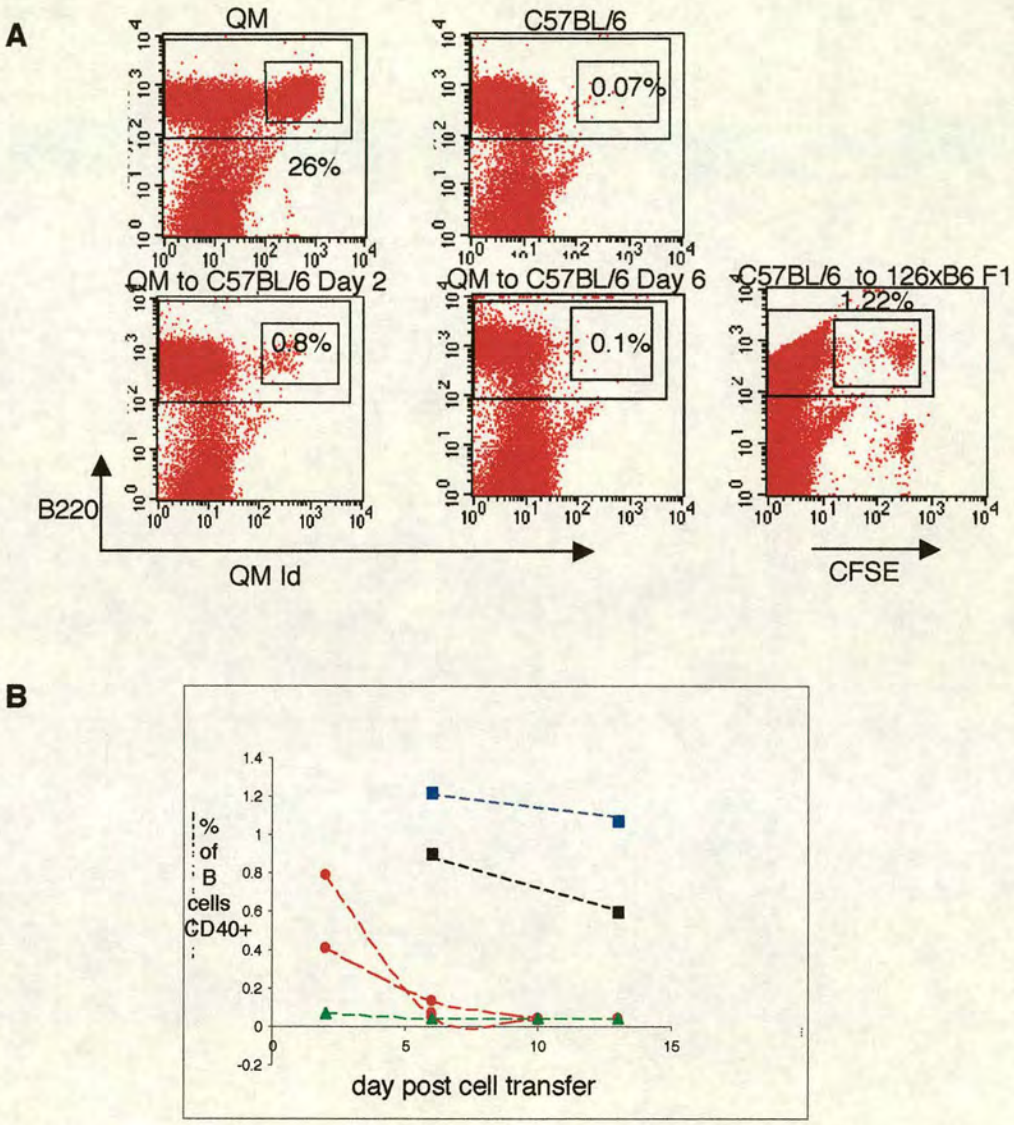
μMT/CD40$^{-/-}$ chimeras received either 1x10^{7} or 5x10^{7} C57BL/6 B cells and were immunised 24 hr later with DNP-KLH. Mice were bled and serum ELISAs performed to detect IgG and IgG1. **A** IgG (left) and IgG1 (right) serum ELISA graphs. Blue lines represent mice that received 5x10^{7} cells whereas red lines represent mice that received 1x10^{7} cells. Negative and positive controls are represented by green and black lines respectively. **B** Spleens were harvested and cells stained for CD40 and B220. B cells were gated and CD40^{+} B cells given as a percentage of total B cells in the table (left). On the right a representative dot plot is shown indicating the B cell (1) and CD40+B cell (2) populations. Gating on B cells (1) CD40 expression is shown in the histogram below.

3.9 Adoptively transferred QM cells are rejected by hosts on a C57BL/6 background

Initially we had attributed the failure to detect transferred QM cells in $CD40^{-/-}$ mice to poor priming resulting from the DC immunisation protocol required (section 3.6). However, similar results were obtained with grafts of QM B cells to the verified $\mu MT/CD40^{-/-}$ chimera system. Transferred cells were not detectable by FACS and GC were not formed (data not shown). Results were replicated in carrier-primed chimaeras. This indicates to us that grafts were being rejected.

In order to be satisfied with results obtained from adoptive transfer experiments we had to be sure that transferred cells QM cells were being activated in the host and forming GCs, the sites of affinity maturation. To do this, grafts need to be stable for at least 3 weeks. To measure rejection of cells QM B cells were transferred to C57BL/6 mice. $1x10^7$ QM splenocytes were transferred and mice immunised i.p. with NP-OVA. Transferred cells were identified by FACS at regular intervals ,with antibodies to B220 and the QM Id. Analogous C57BL/6 to C57BL/6 transfers were performed as a control, in this case cells were marked with the intracellular fluorescent stain CFSE. Numbers of QM cells dropped dramatically post transfer to C57BL/6 mice, some cells were present at day 2 but levels were not above background on day 6 (Figure 3.13). Control grafts were still present on day 14. Although we were aware that QM mice were not fully backcrossed, short term transfers were thought possible.

Figure 3.13 QM cells are not tolerated in C57BL/6 hosts



1x10^7 QM or C57BL/6 splenocytes were transferred to C57BL/6 mice or 129xB6 F1 mice. 24 hr later mice were immunised with NP-OVA. On day 2, 6, 10 and 14 spleens were harvested and splenocytes stained with anti B220 and anti QM Id for analysis by FACS.
In **A** representative FACS plots are shown for 1 of 2 mice In each group. Top: positive and negative control - QM mouse and C57BL/6 mouse. Bottom (L to R): day 2 and day 6 QM to C57BL/6 transfers and C57BL/6 (CFSE) to 129xB6F1.
B The percentage of B cells which were QM or CFSE positive was calculated and is presented graphically. Green symbols represent background QM staining. Red symbols indicate QM to B6 transfers, blue symbols represent C57BL/6 to 129xB6 F1 transfers with QM to 129xB6 F1 transfers represented by black symbols.

Grafts of CFSE labelled QM cells to QM hosts were also rejected (data not shown) indicating that QM mice are not syngeneic, complicating the search for a suitable host.

QM mice were generated on a 129 background and crossed 3 or 4 generations to C57BL/6. As they were not tolerated by mice on a C57BL/6 background we decided to investigate 129xC57BL/6 F1 (128xB6 F1) as prospective hosts for transferred QM cells. Grafts were well tolerated with cells present at levels similar to those of C57BL/6 transfers (Figure 3.13). These mice were used as hosts for the generation of chimaeras in future experiments.

3.10 Construction of a bone marrow chimaera using 129xB6 F1 mice.

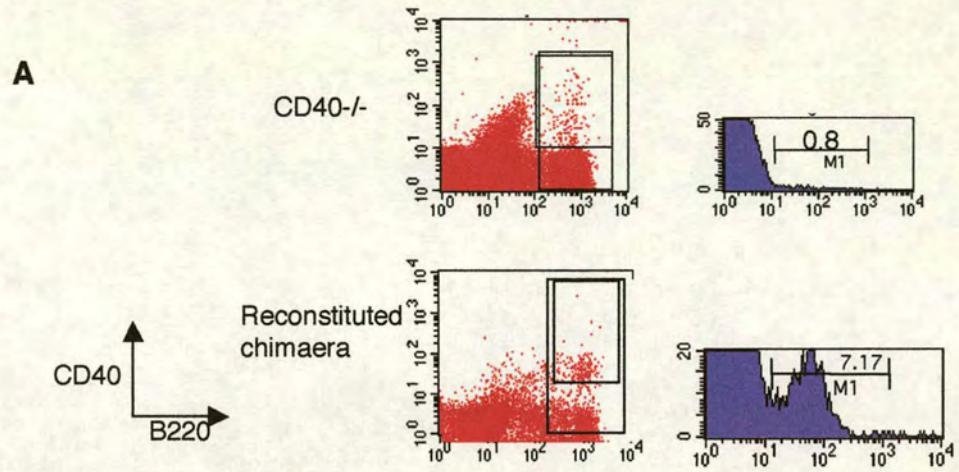
We decided to use 129xB6 F1 mice as hosts for the generation of $CD40^{-/-}$ μMT chimaeras. Although the $CD40^{-/-}$ and μMT mice are both on a C57BL/6 background, during reconstitution from the transferred bone marrow, precursors will be exposed both to 129 and C57BL/B6 antigens on the host stroma. This should tolerise them to QM antigens and avoid the rejection problems encountered with transfers to C57BL/6 hosts.

C57BL/6 mice are irradiated for 47minutes for the generation of chimaeras, as illustrated in figures 3.9 and 3.10, this successfully removes all traces of host B cells and DC. 129xB6 F1 mice require a significantly higher dose than this as leakage of host cells was detected at this level. We were reticent to irradiate for a longer period as prolonged exposure to ionising radiation causes gut lesions in mice. However, if the dose is split in 2, the deterministic effects of

irradiation can be minimised while still effectively depleting host lymphocytes and haemopoietic cells. A titration of radiation dose was performed to find the level at which host tissue is removed without adverse effects to the mice.

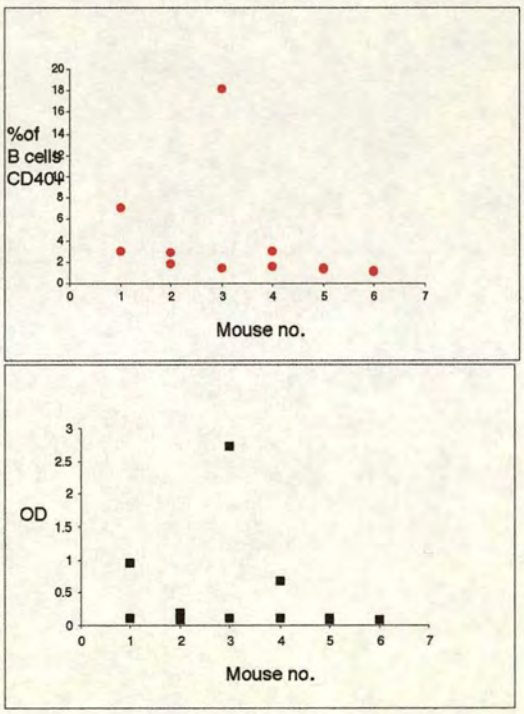
To ensure removal of all CD40 positive cells, after a 2 month reconstitution period, mice were immunised with NP-OVA and blood samples taken for IgG1 serum ELISA. Splenocytes were also analysed by FACS for $CD40^+$ B cells. ELISA results closely mimic FACS data, those mice that have $CD40^+$ B cells make a response that is proportional to the number of cells present (Figure 3.14). Two doses with a 2 hour gap between them was found to be required to completely remove all traces of host B cells. Identical results were obtained from mice that had received either doses of 35+40 min or doses of 35+45 min, it was decided to err on the side of caution and select the higher irradiation dose for generation of 129xB6 F1 host chimeras to ensure complete removal of host lymphoid tissue.

Figure 3.14 Irradiation of 129XB6 F1 host mice for the generation of chimaeras



B

Irradiation condition	Irradiation dose - minutes	% of B cells CD40+
1	50	7.17 3.03
2	55	2.89 1.90
3	60	18.16 1.41
4	35+35	2.99 1.55
5	35+40	1.49 1.34
6	35+45	1.13 1.02
Negative control	CD40-/- host chimaera	1.1 0.8



129xB6 F1 mice were irradiated at various doses and reconstituted with μMT and $CD40^{-/-}$ bone marrow. 8 weeks later mice were immunised with NP-OVA, after a day 10 boost, spleens were harvested and stained for B220 and CD40 expression.
A Representative FACS plots are shown of a $CD40^{-/-}$ mouse and a reconstituted chimaera. Gating on B cells a histogram is shown of $CD40^{+}$ B cells. **B** The percentage of B cells which were $CD40^{+}$ for each of 2 mice per condition was determined and is plotted (top) against irradiation condition, which is shown in the table. Antigen specific IgG1 ELISAs were performed, titres are shown (bottom.)The OD 405 is plotted against irradiation condition.

Discussion

The development of an adoptive transfer system for the transfer of mature splenic B cells has been described. Ideally we wanted to be able to distinguish transferred B cells from host B cells so that they could be visualised by FACS and their response determined by ELISA. The progression of experiments leading to the development of a mixed bone marrow chimaera as an adoptive host is described. Many different types of hurdles were encountered but each in turn lead to a fuller understanding of the requirements for an appropriate, physiological experimental system.

RAG-/- mice as adoptive hosts for transferred B cells.

Initial transfers of C57BL/6 B splenocytes to $RAG^{-/-}$ mice were performed to assess their suitability as a host for mature B cells. Having no mature lymphocytes these mice proffer an easy method of distinguishing transferred cells. All ELISA responses can be attributed to transferred cells and simple markers for mature lymphocytes allow their detection by FACS. We had thought that transferred cells would proliferate and reconstitute the missing lymphocyte compartments in the spleen, just as in normal mice, B cells which die are replaced by new ones from the bone marrow. However it became immediately obvious that mature B cells did not reconstitute the B cell compartment of the spleen, whereas transferred mature T cells almost fully replenished the splenic T cell compartment. In repeated experiments, despite irradiation of the host, transferred B cells failed to proliferate and instead a small but reproducible number of them differentiated into plasma cells. This was

mirrored in the ELISA results where antigen specific serum IgM but not IgG was detected. Immunofluorescent analysis of spleen sections exemplifies these results well; no B cell areas can be seen, but IgM plasma cells are clearly visible along with small T cell areas.

These results seemed difficult to explain without further investigation, however at this time a report by Agenes and co workers was published detailing similar findings in great depth. (182). They find that when resting lymph node B cells are transferred to B cell deficient hosts ($RAG2^{-/-}$) that the number of donor B cells recovered is very low, that they have an activated phenotype, and that serum IgM levels are the same as in normal mice. They attribute this to independent homeostasis of a peripheral pool of activated B cells.

In mice that received $1x10^7$ lymph node cells on day 0, only $1x10^5$ cells were recovered on day 3, however, this improved slightly with $1x10^6$ cells recovered on day10. The number of cells recovered remained stable for 4 months. Cells were found to turn over, however transferred B cells were unable to reconstitute normal B cell numbers. Many of these cells had an activated phenotype suggesting that some donor B cells differentiate from a resting to an active state or that pre-activated cells in the transferred population proliferate. In either case, the surviving population is different from the resting B cell compartment in normal mice. Similar findings have been reported for a mouse with an inducible RAG2 mutation; a small population of activated cells remain while normal B cells disappear with time (184).

These results mean that $RAG^{-/-}$ hosts are unsuitable for the transfer of mature B cells. The reason for this might be that in a lymphopeonic setting it is

important to attain protective immunity. This is done by immediate differentiation to plasma cell rather than reconstitution of the B cell compartment. Or perhaps mature B cells, although they have the ability to divide are lacking other unknown factors that allow the reconstitution of a niche in an immunodeficient host. The importance of the adoptive host is stressed by Freitas:

> "B cell survival is not predetermined by intrinsic properties of the cell but rather it is dependent on the host environment and the presence of other competitor populations "(185)

Many other labs have used RAG deficient mice as hosts for the adoptive transfer of B cells. Much of the work investigating the expression or re-expression of RAG in the periphery involved the transfer of B cells from either RAG2-GFP (143, 149) or RAG-1-GFP (146) fusion gene targeted mice, or mice carrying a bacterial artificial chromosome (BAC) encoding GFP instead of RAG2 (147) to RAG1 deficient mice. We feel that transferred cells should be able to take part in a normal immune response and that this should be ensured by monitoring their response to antigen. Although some information can be determined by transferring cells to RAG deficient animals for our purposes we felt a more physiological system was required.

Other adoptive hosts investigated.

Our results with transfers to RAG $^{-/-}$ hosts led us to investigate the use of other mice, which have their own complement of B cells. However, this can cause other difficulties. If host B cells are present it makes the identification of

transferred cells more complex. We needed a strategy for the detection of the response of transferred cells by ELISA, to assess affinity maturation, and a cell surface marker for their detection by FACS. It is important that transferred cells should be distinguishable from host cells in the event of loss of Id expression. As discussed before there are at least 3 well-documented systems for identification of transferred cells. However, $CD40^{-/-}$ mice were available to us and had the advantage of lacking the cell surface marker but also of being unable to class switch. This allows the detection of the response of transferred cells by class switched isotype ELISA.

$CD40^{-/-}$ mice have the added advantage that CD40 deficient B cells are unable to make TD responses as they cannot receive signals from CD154 on T cells. They do not form GCs, nor do the B cells proliferate in response to antigen. This gives transferred $CD40^{+}$ cells a large selective advantage. This selective advantage helps to counteract the stress cells are put under when manipulated *ex vivo*. Cell sorting limits the number of cells that can be transferred so this selective advantage is beneficial. Adoptively transferred cells are also in the position of being extra cells in a mouse that has already filled all its lymphocyte niches. We were reticent to create space for cells by irradiating hosts as we found this to non specifically activate cells and disturb lymphoid architecture. Hence the use of CD40 deficient mice is ideal.

Although it has been reported that B cells transferred to full immunocompetent hosts are lost soon after transfer (186), we have had modest success, with transferred cells still detectable after 16 days. However, in hosts where other B cells are CD40 deficient $CD40^{+}$ B cells can be detected after 3

months. This led to the development of two systems based on the use of CD40 deficient B cells as compromised competitors for survival factors with transferred cells. The first one relied on the immunisation of $CD40^{-/-}$ mice with $CD40^{+}$ DC as these are required to activate T cells so they can provide costimulation for $CD40^{+}$ B cells. Our second system removed the requirement for external administration of DC, by generating a mouse with DC and B cells compartments provided by different transgenic mice. This allows DC to be CD40 sufficient in the context of CD40 deficient B cells, an ideal host for adoptive transfer of a pure population of transgenic $CD40^{+}$ B cells.

Mixed bone marrow chimaeras have been used by other groups to investigate competition between cohorts of B cells for space during reconstitution (185) . However chimaeras where DC and B cell compartments are from different sources have not been documented. They were developed in our lab initially, to allow dissection of the factors involved in T cell migration to B cell follicles (S. Fillatreau and D. Gray in press). They have also been used to investigate cognate interaction between B and T cells where B cells, but not DC, are class II deficient. The real benefit of this system is that it allows the isolation of a genetic deficiency to the B cell compartment alone, allowing dissection of the role of B cells in the context of a functioning immune system.

At this stage our experiments were complicated by the rejection of QM cells by hosts on the C57BL/6 background. Although we were aware that the QM mice were not fully backcrossed we had though that grafts would be stable long enough for our purposes but this proved not to be the case.

This meant that as well as providing a means for the cells to be distinguished by FACS and their response by ELISA, the adoptive host needed to accept QM grafts. The use of 129xB6 F1 hosts means that transferred bone marrow cells used for reconstitution are educated in the presence of stroma expressing antigens of both 129 and C57BL/6 origin. Surprisingly these mice are particularly radio resistant and it took some time to find irradiation conditions that removed all host cells. However, these mice were tolerant of QM grafts.

Chimaeric hosts provide an environment for the activation, selection and expansion of transferred $CD40^+$ B cells. Cells are able to class switch and form GCs. Their antibody responses can be distinguished from those of the host as they can class switch and produce IgG and its subclasses. Cells can also be visualised by staining with an anti-CD40 antibody and analysed by FACS. This chapter details the development of this system as a suitable adoptive host for our requirements. In subsequent chapters experiments investigating the response of transferred cells are described.

Chapter 4 Receptor revision in QM B cells

Introduction

In chapter 3 the development of an adoptive host for the receipt of a pure population of QM B cells is described. In this chapter a series of experiments to investigate whether a pure population of splenic QM Id^+ cells might produce a serum antibody response to antigens other than NP, the hapten recognised by the site directed BCR is described. It has already been reported that centrally edited Id^- cells in the QM mouse can provide neutralising antibody responses to several viruses (180). We have also shown that they can provide a response to model antigens (section 3.2, 3.3). We wanted to know whether *de novo* rearrangements of the BCRs of mature splenic QM B cells could provide a serum antibody response to other antigens.

QM mice were chosen as a source of transgenic cells specifically because they recognise the hapten NP. Haptens when conjugated to protein carriers elicit a TD immune response which has been well characterised (187, 188). Since haptens are small and only contain one epitope, the antibody response can be measured in ELISA by conjugation of the hapten to a different carrier, allowing an uncomplicated measure of serum antibody titres. The other site directed transgenic mouse used in this study, 3.83KI (Chapter 5), requires the use of a complex antigen – M13 bacteriophage, the response to which is less well defined.

The QM mouse undergoes secondary rearrangement of its antibody heavy chain. It is the heavy chain that provides the specificity for antigen as all

combinations of site directed transgenic heavy chain with endogenous lambda light chains result in NP specificity (178). There is strong selective pressure in this mouse for the expansion of other specificities. This is substantiated by the ~25% of B cells in the spleen that no longer express the idiotypic antibody due to central editing. As the QM heavy chain is known to undergo rearrangement the QM model is a good choice for investigating the effects of receptor revision at the heavy chain locus on affinity maturation.

In experiments performed by Nemazee and co-workers receptor revision in 3.83 transgenic mice was detected in response to antigen recognised with low affinity by the transgenic BCR. High affinity or non-binding antigen did not induce receptor rearrangement (137). We have adopted this antigen system in our investigation of affinity maturation in 3.83 mice (Chapter 5). However, there is not a known hierarchy of antigens recognised by the QM receptor. In a recent publication (176) pNP (p-nitrophenyl acetyl) was used as an antigen recognised with low affinity by the QM receptor. In the experiment described here it was decided to use the homologues of NP: DNP and TNP, as potential low affinity antigens. TNP has been previously used as an antigen recognised with low affinity by the QM BCR (Gaspal and Lane poster presentation at Keystone B cell symposium 2001). No reactivity to TNP or DNP was detected by ELISA using serum from NP immunised C57BL/6 or QM mice, indicating that if there is any cross reactivity between antigens that it is very low. Consequently these antigens (TNP and DNP) are ideal candidates for induction of receptor rearrangement.

In chapter 3 the development of a mixed bone marrow chimaera adoptive transfer system was described. Here, the results obtained from the

transfer of mature sorted QM Id^+ B cells into chimaeric hosts are reported. Mice were immunised with NP, DNP or TNP and serum antibody responses measured by ELISA. Strong antibody responses were detected to NP, however, there was no response to DNP. A small, delayed response to TNP was detected in some mice. The implications of these findings are discussed.

Results

4.1 QM cells grafted to chimaeric hosts undergo cell division when immunised with NP-OVA.

It is critical to the experiment that adoptively transferred cells should be responsive to antigen and capable of normal B cell immune responses. To determine whether transferred cells become activated after immunisation of host mice, we examined their cell division using the fluorescent stain CFSE. This dye stably binds cytoskeletal actin following activation by cellular esterases. When a cell divides CFSE is apportioned equally between the 2 daughter cells, resulting in halving of the fluorescence, consequently cell division can be examined by FACS (189, 190). QM and C57BL/6 splenocytes ($1x10^7$) or sorted QM Id^+ B cells ($1x10^6$) were CFSE labelled and transferred to 129xB6 F1 host chimaeras reconstituted with $CD40^{-/-}$ and μMT bone marrow (for simplicity these will be referred to as chimaeras in the remainder of this chapter). Mice were immunised 24hr later with NP-OVA alum. After 7 days

mice were sacrificed and splenocytes analysed by FACS (Figure 4.1).

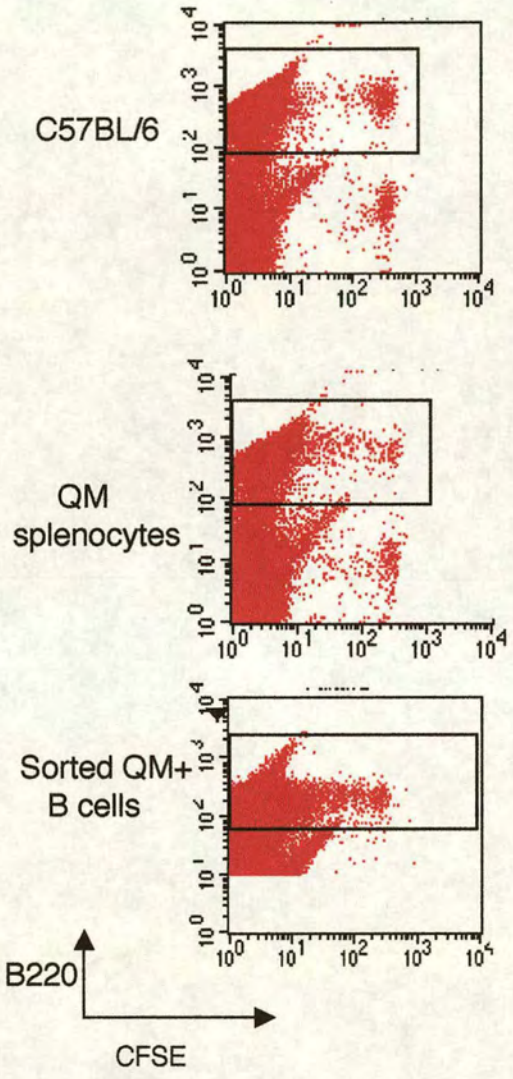
QM cells divided well with many having gone through at least one cell division, this was even more impressive on day 14 with very few cells maintaining full levels of fluorescence (not shown). Sorted B cells behaved similarly to splenocytes, indicating that sorted cells maintain the ability to respond to antigen. In contrast, much fewer C57BL/6 B cells underwent cell division, this is unsurprising as few of them should recognise NP. It is difficult to estimate exactly how many divisions cells have completed, as after about 3 rounds the loss of fluorescence causes cells to blend into host cells, however, we are content that transferred QM cells respond to antigen in a normal fashion.

4.2 Transferred QM cells form germinal centres which can be detected by immunofluorescence

It is important that transferred $CD40^+$ B cells are able to form GC in the chimera system. The formation of GC is critical in any system designed to investigate affinity maturation, and other processes in the normal B cell response to antigen. In GC, high affinity B cells are selected on the basis of competition for antigen captured on FDC networks and expanded. Also, RAG expression in the periphery has been localised to GC and to cells with a GC like phenotype (130).

To confirm that GC can form in this system, $1x10^7$ QM or C57BL/6 splenocytes were transferred to chimaeras. Mice were immunised with NP-OVA 24hr later and spleen sections taken for immunofluorescent analysis on day 7 and 14.

Figure 4.1 Division of QM cells in chimaera



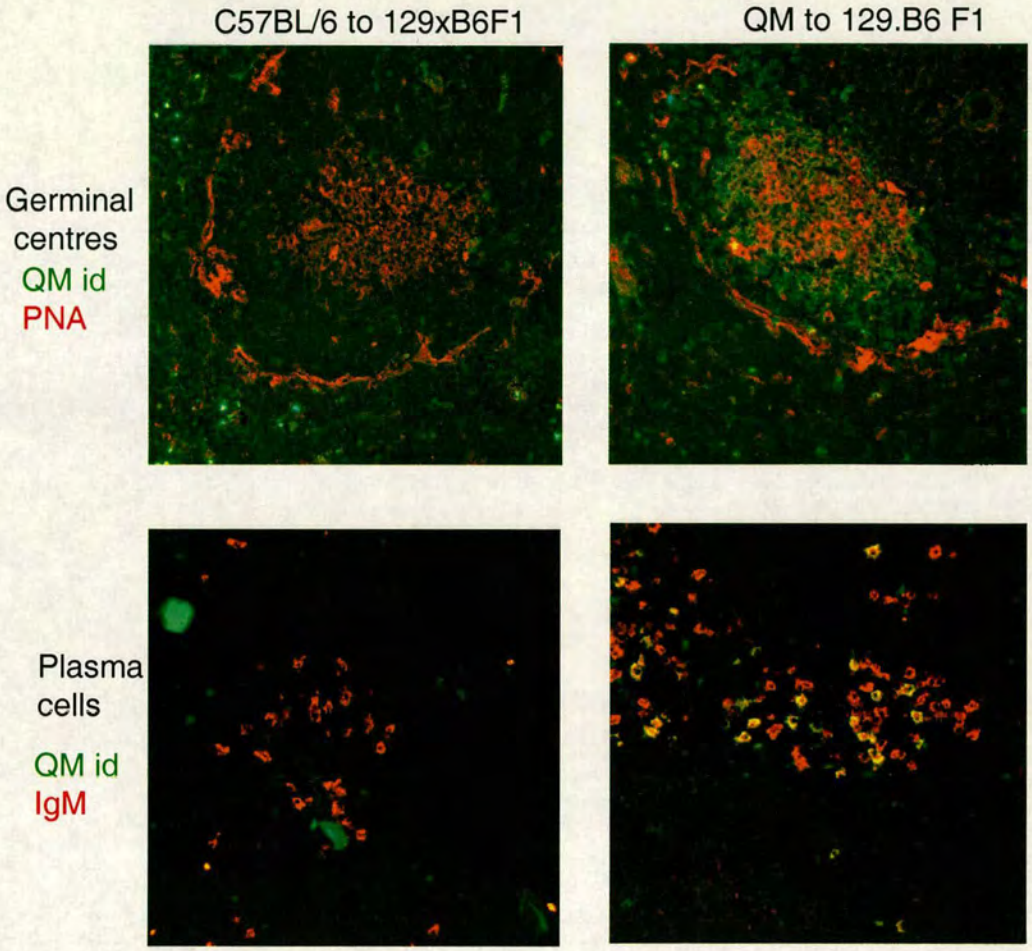
1x10^7 C57BL/6 or QM splenocytes or 1x10 ^{6} sorted QM$^+$ B cells (all CFSE labelled) were transferred to 129xB6 f1 host chimaeras. 24hr later mice were immunised with NP-OVA. After 7 days spleens were harvested and cells stained with anti-B220 conjugated to PE before analysis by FACS. Shown, from top to bottom are mice that received C57BL/6 splenocytes, QM splenocytes and QM sorted B cells. For the mice that received splenocytes 200,000 events in the lymphocyte gate were collected, for the mice which received B cells 500,000 events were collected gating on both lymphocytes and B cells.In all cases CFSE is on the X axis and B220 on the Y axis Data shown is representative of two mice per group on each day. Similar results were obtained on Day 14.

GCs were not detected on day 7 but on day 14 GC numbers similar to control levels were detected. The anti QM Id antibody is faintly visible on sections and it is possible to discern whether GC are composed of QM Id^+ cells or not. When QM splenocytes were transferred and mice were immunised with NP all GC were QM Id^+, in contrast all GC in the mice that received C57BL/6 cells were QM Id^-. QM Id^+ plasma cells are also clearly visible (Figure 4.2), indicating differentiation of transferred cells into plasma cells.

4.3 Transferred, unsorted QM splenocytes can mount a response to two chosen antigens.

We have shown that centrally edited cells can mount a response in the QM mouse. As a final control we wanted to show that centrally edited cells were activated and expanded after transfer to chimaeras, and that these cells could mount a serum antibody response detectible by ELISA. This would confirm that the selection and expansion of a small number of antigen specific cells is possible and that our detection method is sensitive enough to measure an antibody response from these cells. In turn this would confirm that if cells were to undergo revision and provide a useful specificity, that the machinery is present to incorporate them into an immune response and that we could detect this response.

Figure 4.2 GC formation by QM cells in chimaeras



$1x10^7$ C57BL/6 or QM splenocytes were transferred to 129xB6 F1 host chimaeras. Mice were immunised after 24hr with NP-OVA and14 days spleens harvested and sections cut for immunofluorescent analysis. Sections were stained with PNA Texas red and anti-QM Id FITC (top), to stain idiotype positive GC,or IgM texas red and anti QM Id FITC (bottom) to illuminate QM positive plasma cells. On the left C57BL/6 transfers to chimaeras are shown with QM transfers on the right.

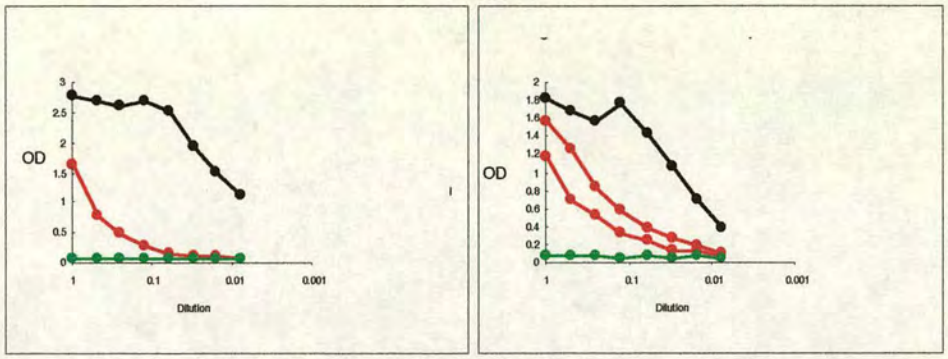
QM splenocytes were isolated and $1.5x10^7$ cells injected intravenously (i.v.) to chimaeric hosts. Typically 25% of the QM spleen is comprised of B cells, therefore we estimate that mice received ~$3.75x10^6$ B cells. Mice were immunised i.p. with DNP-OVA or tetanus toxin RCF, blood samples taken at weekly intervals, and serum ELISAs for all isotypes performed. IgG1 results are shown as a representative example, however, antibody of all IgG isotypes was made, although the levels are about half the magnitude of that of a wt mouse. FACS was also carried out to detect expansion of cells that were QM Id^-, and $CD40^+$. Immunofluorescent analysis of spleen sections was performed to detect the presence of GC (Figure 4.3).

In QM mice around 25% of the B cells in the spleen are Id negative, this is very consistent (our observation). In the chimaeras that received splenocytes this number increases to 56% in the RCF immunised mouse and to 45% and 52% respectively in the 2 DNP immunised mice, this is most likely caused by expansion of Id negative cells rather that receptor revision of Id positive cells. This is probably in response to clonal expansion of selected cells following immunisation, however, we cannot be sure that in the absence of immunisation, Id negative cells would not expand.

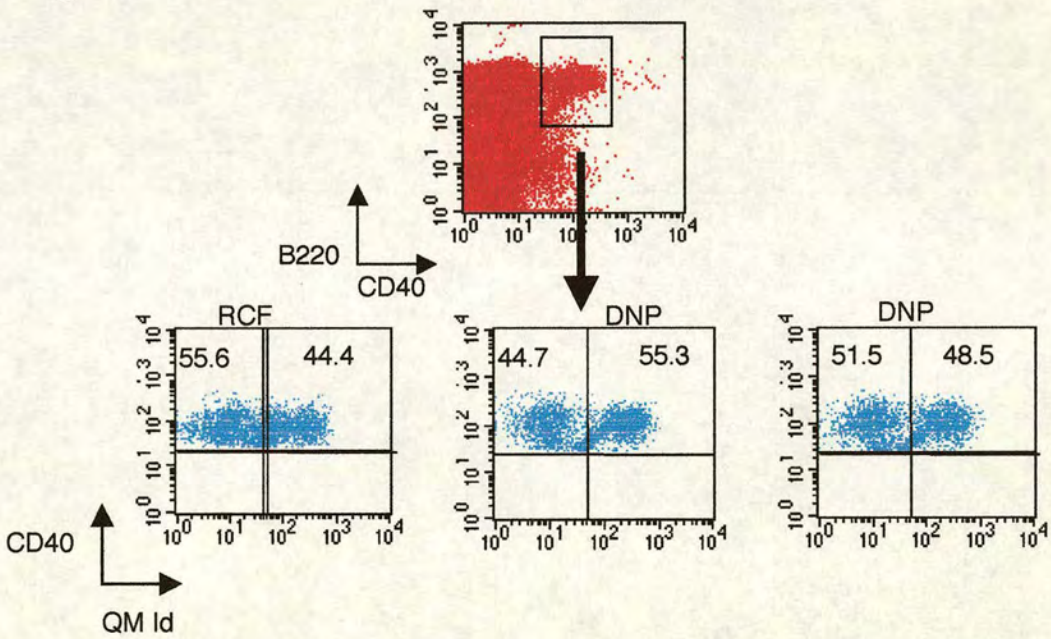
Spleen sections were stained with PNA and anti-QM Id to quantify GCs. The density of GC in experimental mice was the same as that of a wt mouse. All GC were QM Id^- indicating that they were formed by centrally edited cells.

Figure 4.3 Antibody responses of unsorted QM splenocytes when transferred to chimaeric hosts.

A



B



1.5x10^7 QM splenocytes were transferred to three chimaeras. 24hr later 1 was immunised with RCF and 2 with DNP-OVA. Blood samples were taken every 7 days and on day 21 spleens were harvested and 3 colour FACS analysis performed.

A ELISAs showing levels of RCF(left) and DNP (right) specific IgG1 antibody. Experimental mice are depicted in red with positive and negative controls in blackand green respectively.

B Splenocytes were stained with B220 PerCP, CD40 PE and QM Id FITC. Gating on CD40$^+$ B cells (top) mice were analysed for QM Id expression. Numbers indicate the percentage of CD40$^+$ B cells which are Id$^+$ or Id$^-$

These results confirm that selection and expansion of QM Id $^-$ cells in response to non-NP antigens can happen in this system. Even when the majority of CD40$^+$ B cells recognise NP, mice can make a response to DNP and RCF, two selected model antigens.

4.4 A pure population of QM Id positive splenic B cells can respond to NP in adoptive hosts.

We have shown that a mix of QM Id positive and negative cells can respond to other antigens in an adoptive host, hence verifying our system. To see if a pure population of QM cells can alter its specificity by secondary V(D)J recombination and provide a response to other antigens, cells were sorted by flow cytometry before transfer to chimaeric hosts. A population of ~99% pure QM Id $^+$ B cells was isolated as described in section 3.4. Between 2 and 3x10^6 cells were transferred to chimaeras. To account for the small percentage (~1%) of contaminating Id negative cells, sort purity was assessed after each sort on a FACS Calibur flow cytometer and the requisite number of sorted Id $^-$ cells transferred to control mice.

Mice were immunised with NP or either DNP or TNP, homologues which may be recognised at low affinity by the transgenic receptor and boosted every 7 days. All antigens were alum precipitated and 10^9 *Bordetella pertussis* was added. Further mice were immunised, which did not receive any transferred cells, to control for any host response. Due to the limited yield of cells after sorting, the experiment was split into 3 groups with each group

having at least one NP immunised mouse as a control for cell survival post transfer. In each group, for each mouse that received QM Id $^{+}$ cells, a control mouse received QM Id $^{-}$ cells (Table 4.1). This was to ensure that any response we detected could be attributed to *de novo* rearrangements rather than to existing centrally edited cells in our sorted population. Blood samples were taken and mice were boosted at weekly intervals up to day 21 when mice were sacrificed. Serum ELISAs were performed to assess the levels of IgG and its subclasses secreted by transferred cells (host cells can only produce IgM). Spleen cells were analysed by FACS to ensure the presence, and assess the composition, of transferred CD40^{+} B cells.

Table 4.1 Experimental organisation.

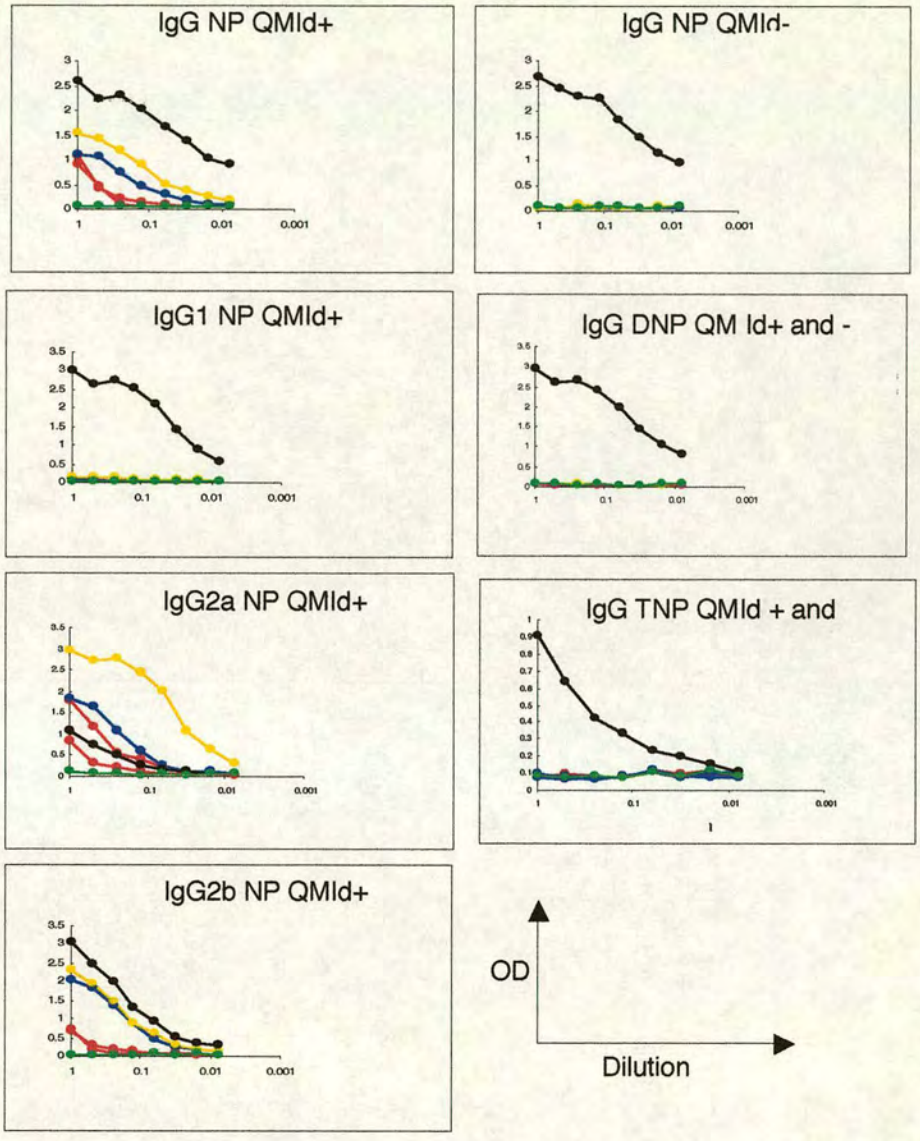
Cell type transferred/ Antigen	Expt No1	Expt No. 2	Expt No. 3
QM Id^{+} NP	2NP	1NP	1NP
QM Id+ DNP or TNP	2TNP	2TNP	3DNP
QM Id -	2NP 2TNP	1NP 2TNP	1NP 3DNP
No cells	1NP 1TNP		2NP 2DNP

At day 7 all mice make antigen specific IgM (data not shown) indicating successful immunisation with all antigens. All NP immunised mice that received QM Id^+ cells make an IgG response, this is half the magnitude of that made by a wt mouse on day 14 (Figure 4.4) and indicates that transferred cell survive and respond. This IgG response is comprised of IgG2a and IgG2b, with no IgG1 being secreted at this time. None of the NP immunised mice, which received QM Id^- cells or no cells, have any antigen specific IgG indicating that the response can be attributed solely to QM Id^+ cells. There is no IgG response of any type to TNP or DNP indicating a failure of transferred cells to make a serum response to these antigens.

4.5 On day 14 serum anti-NP responses are similar to those of a wt mouse

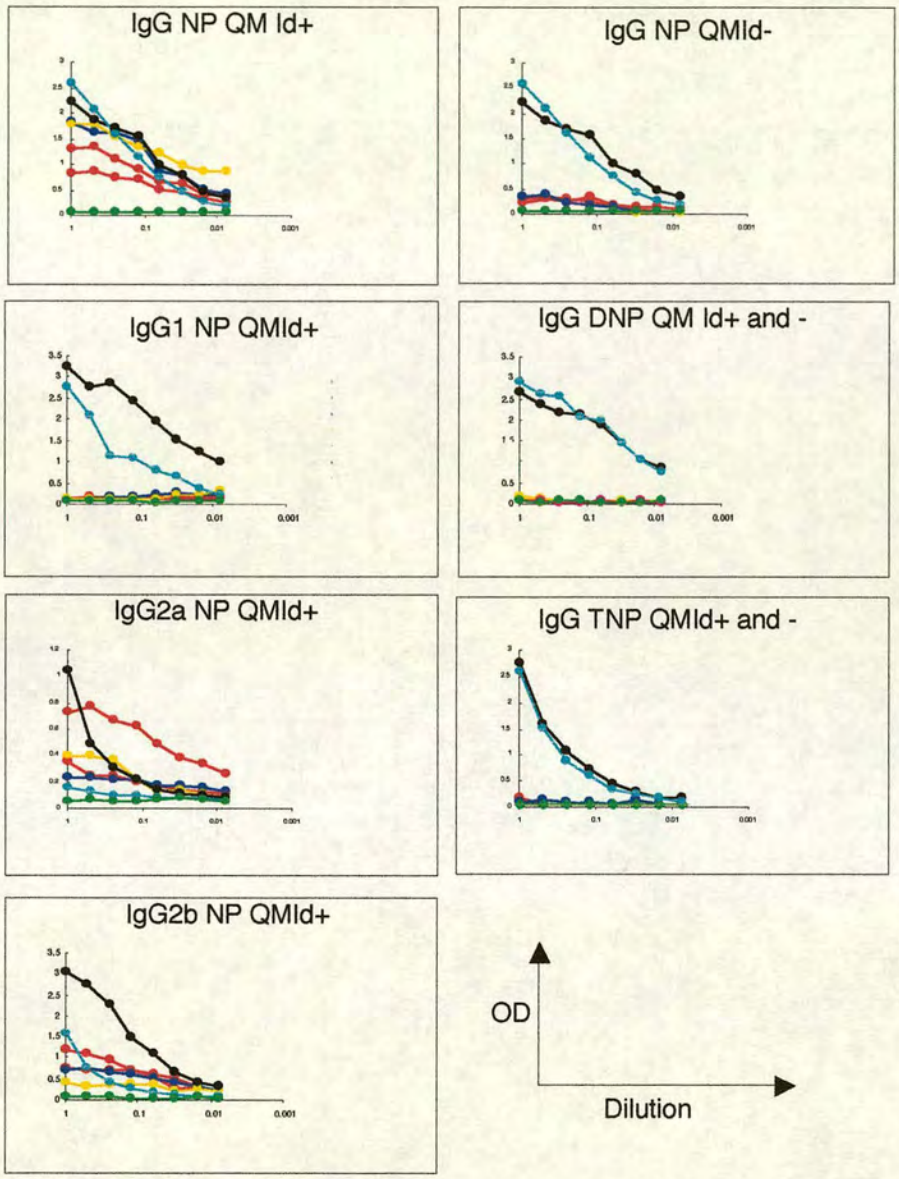
At day 14 the strength of the IgG response is similar to that of a wt mouse. There is the same contribution from IgG subtypes as at day 7 and there is still no IgG1 (Figure 4.5). DNP and TNP immunised mice still have no antigen specific IgG. Interestingly, however, in the NP immunised mice a low level of IgG can be detected from mice that received ~1% QM negative cells to control for cell sorting accuracy. Mice that received no cells have background levels indicating that the response is not coming from the host but from the transferred cells. When sorting QM B cells, it is difficult to obtain a purity level above 99%. 1% sorted QM negative cells are used to control for this, however, the QM Id^- cells themselves are not 100% pure and contain about 1% QM positive cells.

Figure 4.4 Responses of QM cells day 7 post transfer



Between 1 and 3x10^6 QM$^+$ B cells were transferred to chimaeras, control mice received either no cells or the requisite number of sorted QM $^-$ cells. Mice were immunised with 100μg of either NP, DNP or TNP conjugated to OVA with 10^9 pertussis. Mice were bled on day 7 and serum ELISAs performed for IgG and its subtypes. On the left are shown NP immunised mice that received QM $^+$ cells from top to bottom IgG, G1, G2a and G2b. Mice in different experiments (cells sorted on different days) are coloured red, blue and yellow the positive and negative controls are black and green respectively.
On the right are IgG elisas from the top NP immunised mice that received negative cells (7mice), DNP immunised mice that received positive or negative cells (8 mice) and TNP immunised mice (9mice) as they are all negative individual lines cannot be distinguished. Positive controls are depicted in black

Figure 4.5 Day 14 serum ELISA data



Day 14 serum antibody responses were determined by ELISA. On the left are NP IgG responses in mice that received QM^+ cells, from top to bottom: IgG, G1, G2a, G2b. Mice in different experiments (cells sorted on different days) are coloured red, blue and yellow, the positive and negative controls are black and green respectively. Depicted in pale blue is the day 14 response of chimaeras reconstituted with wt cells to control for the irradiation and reconstitution process

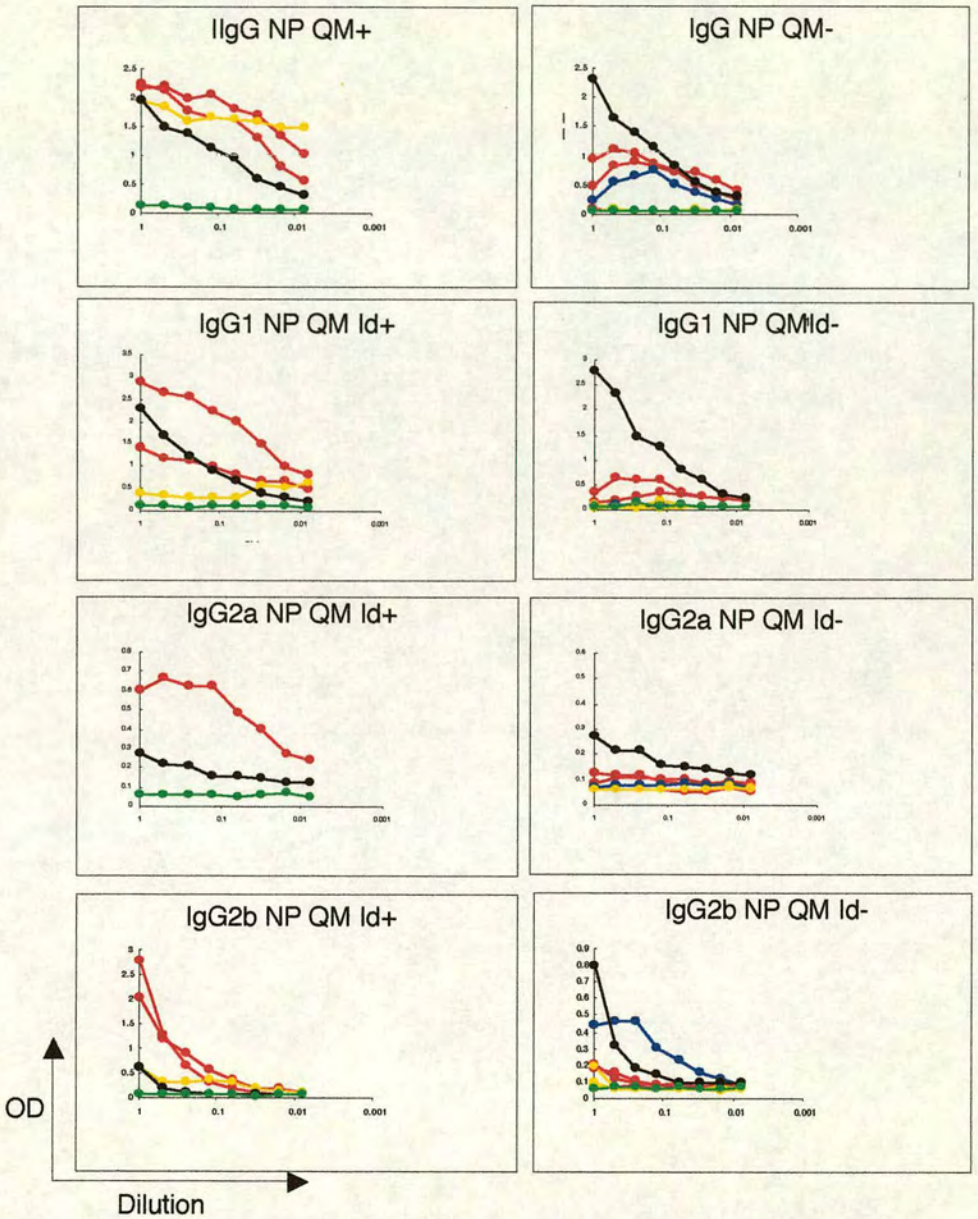
On the right are IgG elisas, from the top NP immunised mice that received negative cells or no cells (7mice), DNP immunised mice that received positive, negative or no cells (8 mice) and TNP immunised mice that received positive , negative or no cells (9mice) -as they are all negative individual lines cannot be distinguished. ELISA positive controls are depicted in black, in pale blue is the day 14 response of chimaeras reconstituted with wt cells.

Although only tens of thousands of negative cells were transferred to each mouse and only about 100 of these cells (1%) will have NP specificity we can still detect their response our assay, indicating that the response of a small number of cells can be detected in this system. However, it also indicates that the mice that received QM Id $^-$ cells as a control for sort purity should be treated cautiously as a marker of the response of contaminating QM Id $^-$ cells in the QM positive population. On day 14 another control is included; 129.B6 F1 host mice were irradiated and reconstituted with C57BL/6 bone marrow to control for the irradiation and reconstitution process. Levels of IgG are similar to those of a C57BL/6 mouse indicating that mice recover fully from irradiation and reconstitution and that it does not impair the immune response.

4.6 At day 21 a small response to TNP can be detected in mice that received QM Id positive cells but not in control mice

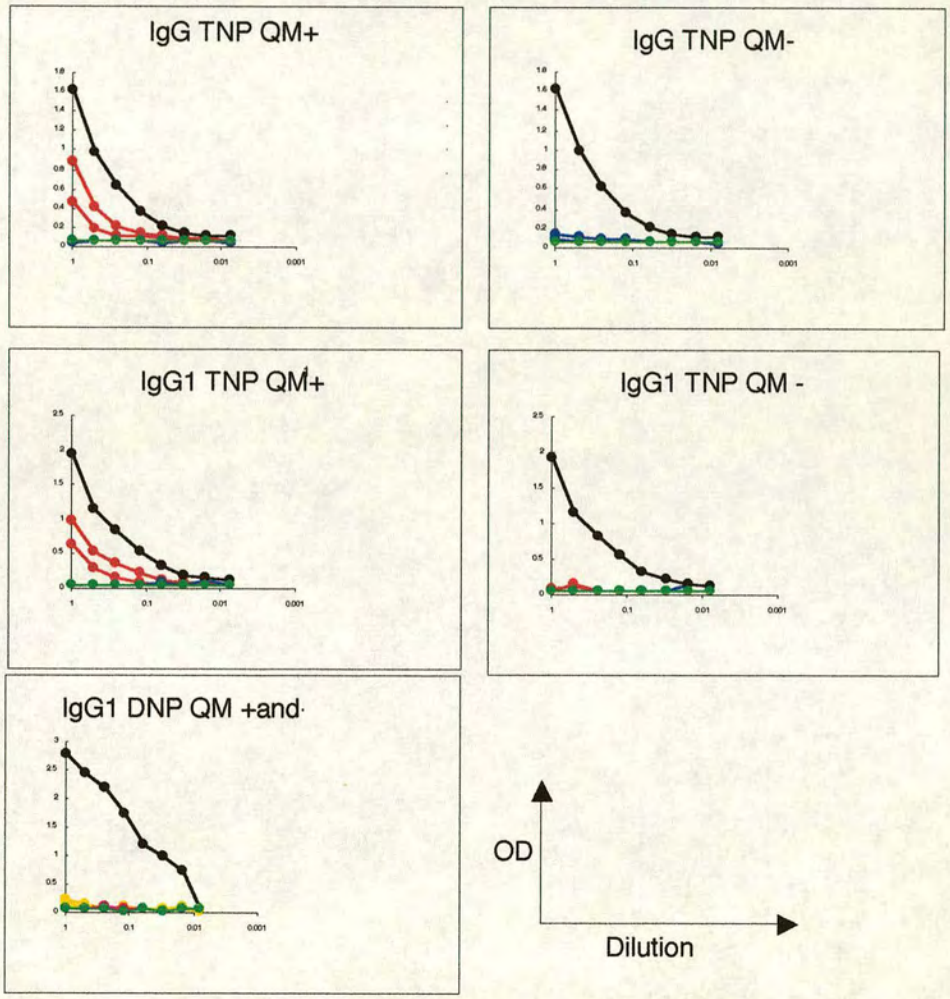
By day 21 the NP response has surpassed that of a wt mouse and the composition has shifted (Figure 4.6). IgG1 is now the major contributing IgG subclass. The response of the mice that received QM Id $^-$ cells is also more clearly visible, with IgG1 and IgG2b being the major contributing isotypes. Again, there is no contribution from host cells of the chimaera, as indicated by a lack of response in immunised chimaeras that did not receive any cells. Unexpectedly, although there is no detectible response to DNP, there is a small IgG response to TNP from 2 mice in one group (Figure 4.7). This response is entirely comprised of IgG1; all other isotypes were absent.

Figure 4.6 Day 21 NP responses in chimaeras with transferred QM cells



Mice were bled on day 21 and serum ELISAs performed for IgG and its subtypes. On the left are shown NP immunised mice that received QM $^{+}$ cells. On the right are mice which received QM negative cells or no cells and were immunised with NP. From top to bottom IgG, G1, G2a and G2b. Mice in different experiments (cells sorted on different days) are coloured red, blue and yellow the positive and negative controls are black and green respectively.

Figure 4.7 Day 21 DNP and TNP responses.



Mice were bled on day 21 and serum ELISASs performed for IgG (top) and IgG1 (below). On the left are shown TNP immunised mice that received QMId $^{+}$ cells. On the right are mice that received QMId $^{-}$ cells, or no cells and were immunised with TNP. At the bottom, day 21 DNP results from mice that received positive, negative, or no cells are shown (8 mice). Mice in different experiments (cells sorted on different days) are coloured red, blue and yellow the positive and negative controls are black and green respectively.

These mice received QM Id^+ cells, there is no response from mice that received either QM Id^- cells, or no cells, indicating that it has been most likely generated by QM Id^+ cells. However, this response is only seen in one of two groups immunised with TNP - 2 out of 4 mice. We appreciate that group numbers could be larger, however, as discussed before, this was made difficult by the low yields achieved when sorting QM cells. This is compounded by the number of control mice employed.

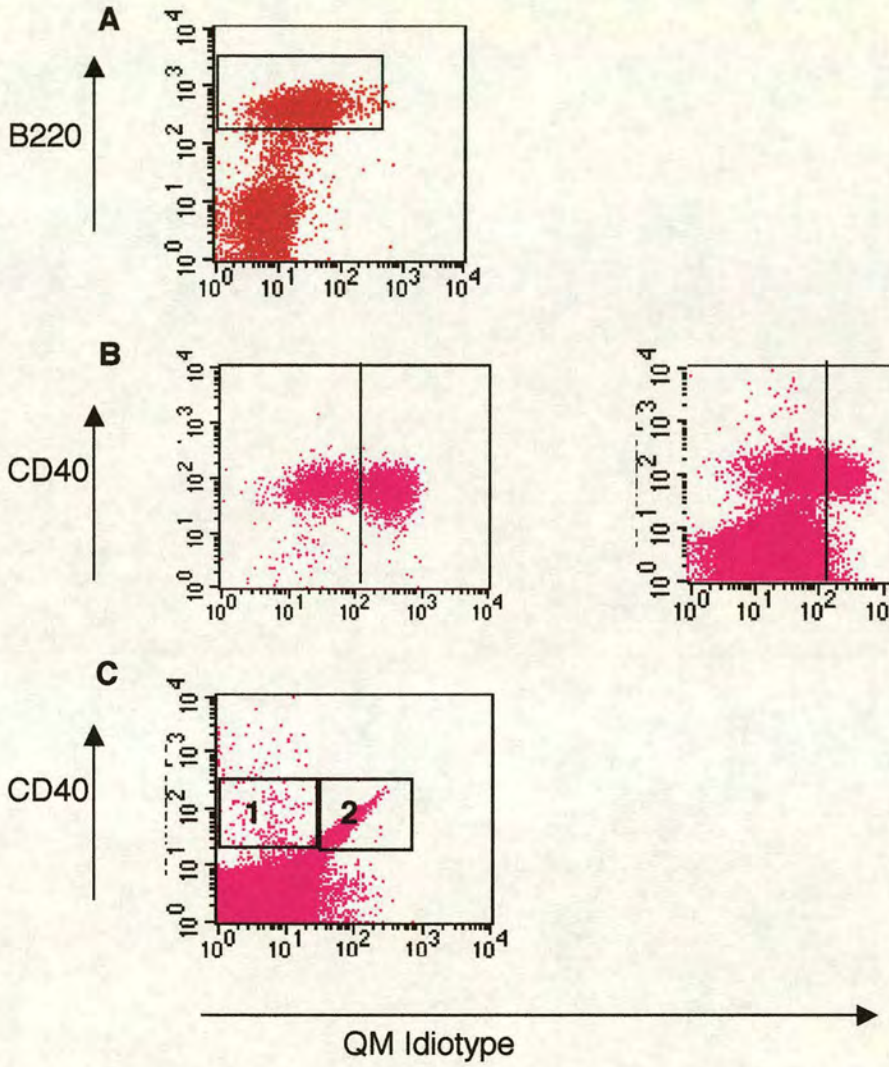
The response to TNP detected, could have been generated by somatic hypermutation or receptor revision. Crossing QM mice onto a to RAG deficient background would make secondary rearrangements impossible, allowing a comparison to be made between the responses of cells that can revise and those that cannot. QM mice cannot be crossed to RAG deficient mice as they require RAG to rearrange the endogenous lambda light chain. Consequently there is no way of ruling out either mechanism in the QM system. This is discussed further in the discussion. In our system B cells are marked with CD40 and with the QM Id, thus in the event of loss of the Idiotypic receptor (by secondary rearrangement or otherwise) transferred cells can still be distinguished from those of the host. Analysis of the composition of $CD40^+$ B cells by FACS is presented in the next section.

4.7 Detection of transferred cells is complicated by high levels of serum Id positive Ig.

QM cells transferred to the chimaeras can be distinguished on the basis of QM Id expression but also on the basis of CD40 expression. This means that if cells were to revise their BCRs and loose Id expression, they should still be distinguishable from host $CD40^-$ B cells because they express CD40. This should show whether receptor revision had occurred. On day 21 spleens were harvested from chimaeras and analysed by three colour flow cytometry for the presence of transferred cells. Splenocytes were stained for the B cell marker B220, for CD40 and QM Id expression. To eliminate host DCs, which can also express low levels of B220 and are $CD40^+$, FACS plots were gated on $B220^{hi}$ B cells and analysed for Id and CD40 expression.

In previous transfer experiments to C57BL/6, or to 129xB6 F1 host chimaeras, QM Id^+ B cells could be easily distinguished from host cells at day 7 or day 14. However, in this experiment, in which mice were boosted multiple times to increase our chances of detecting a response, 3 problems were encountered which hindered our detection of Id expression. Firstly, host $CD40^{-/-}$ B cells (but not T cells) shifted on staining with anti Id antibody making it difficult to distinguish truly Id^+ cells from background (Figure 4.8A). We attribute this to Fc receptor mediated binding of Idiotypic antibody, which is present at high levels within the host. This may be subsequently detected on staining *ex vivo* with the anti Id antibody.

Figure 4.8 Problems encountered when attempting to identify whether transferred CD40$^+$ cells still expressed the idiotypic receptor.



Chimaeras that received transferred cells and were immunised with NP-OVA, boosting weekly, were killed on day 21 and spleens harvested. Splenocytes were stained for B220, QM Id and CD40 expression and analysed by FACS.
A.Splenocytes gated on live lymphocytes stained with B220 and QM Id shows shift of host B cells with anti- Id.
B. Gating on lymphocytes and B220 ^{hi} cells (gate in A) CD40 and QM Id expression is examined in a QM mouse (left) and a chimaera that received sorted QM Id$^+$ CD40$^+$ B cells (right). The vertical line indicates the cut off between QM positive and negative cells in a QM mouse. All of the CD40+ cells in the chimaera should also be QM $^+$ however expression is down regulated.
C. A CD40$^{-/-}$ mouse gated on lymphocytes and B220 ^{hi} cells illustrating the autofluorescence encountered in the spleen, making enumeration of putative Id positive and negative cells impossible (boxes 2 and 1 respectively).

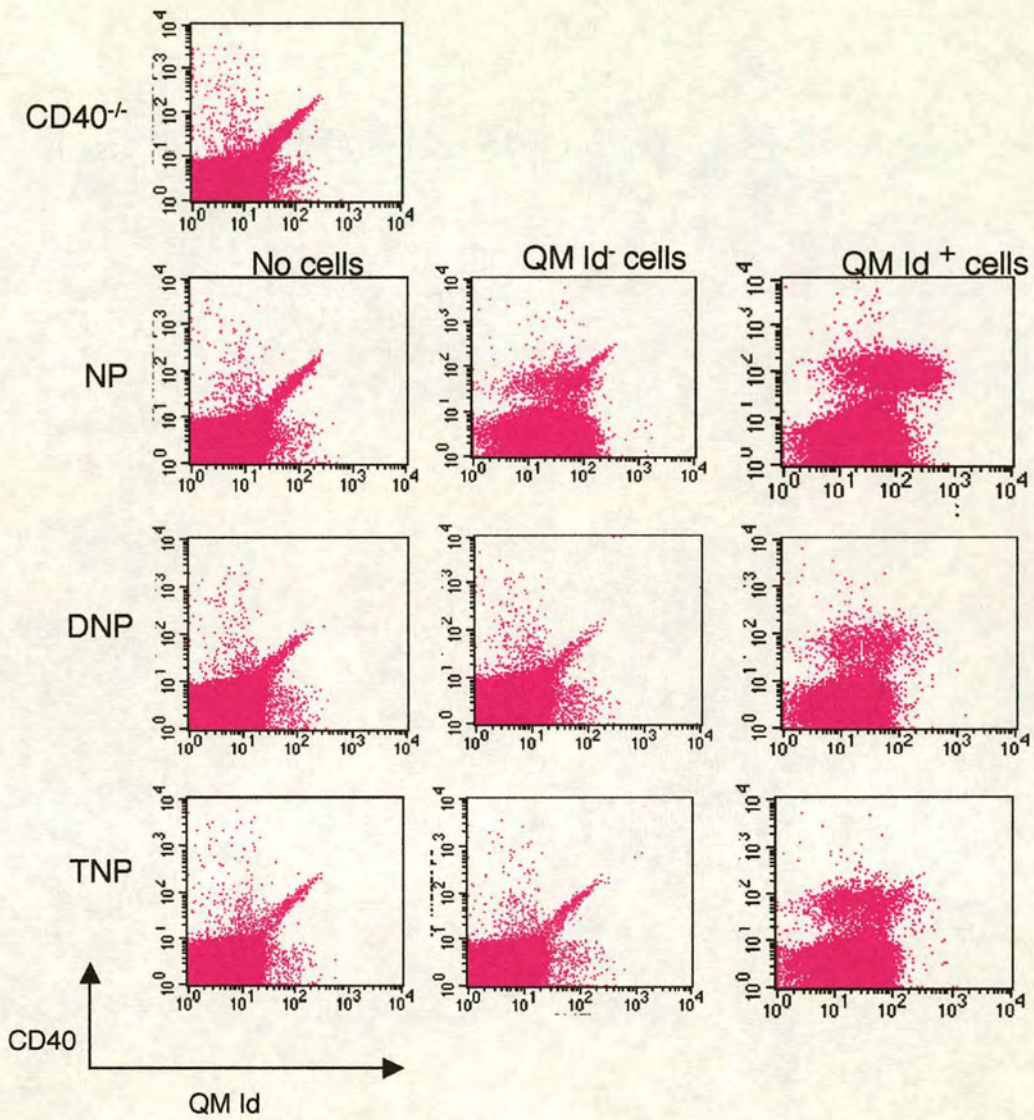
This phenomenon has only been detected in mice that have high levels of NP specific antibody from transferred cells.

Secondly transferred QM Id $^{+}$ cells appear less brightly stained than they were when they were sorted, compounding the previous problem. This may be attributible to down regulated surface Ig due to cell activation (Figure 4.8B). Thirdly, autofluorescent cells in the spleen, which lie in the same position as CD40^{+} Id positive cells on FACS dot plots, make quantitative analysis of transferred cells very difficult (Figure 4.8C). These autofluorescent cells are only noticeable when large numbers of events are collected, however, it is necessary to collect large numbers to detect rare transferred cells. These results are commented on further in the discussion.

4.8 Transferred cells are still present on day 21 and have expanded in NP immunised mice.

Despite that fact that we cannot make any conclusions on Id expression of transferred cells in these mice, transferred cells can still be detected on the basis of CD40 expression. Representative FACs plots from each group of mice are shown in Figure 4.9. For each antigen (NP, DNP and TNP) there is a group of mice that received positive cells, negative cells or no cells and there are between 3 and 5 mice in each group.

Figure 4.9 CD40 positive cells in chimaeras on day 21



On day 21 spleens from chimaeras were harvested and stained for B220, CD40 and QM Id. Plots shown are gated on live lymphocytes and B220 ^{hi} cells to eliminate DCs. At the top (L) is the CD40$^{-/-}$ control illustrating autofluorescence in the spleen. From left to right in columns are representative mice of each of the groups which received; no cells, QM Id $^{-}$ cells and QM Id $^{+}$ cells. In rows across, from the top are mice immunised with NP, DNP and TNP respectively. QM staining is on the X axis and CD40 staining on the Y axis.

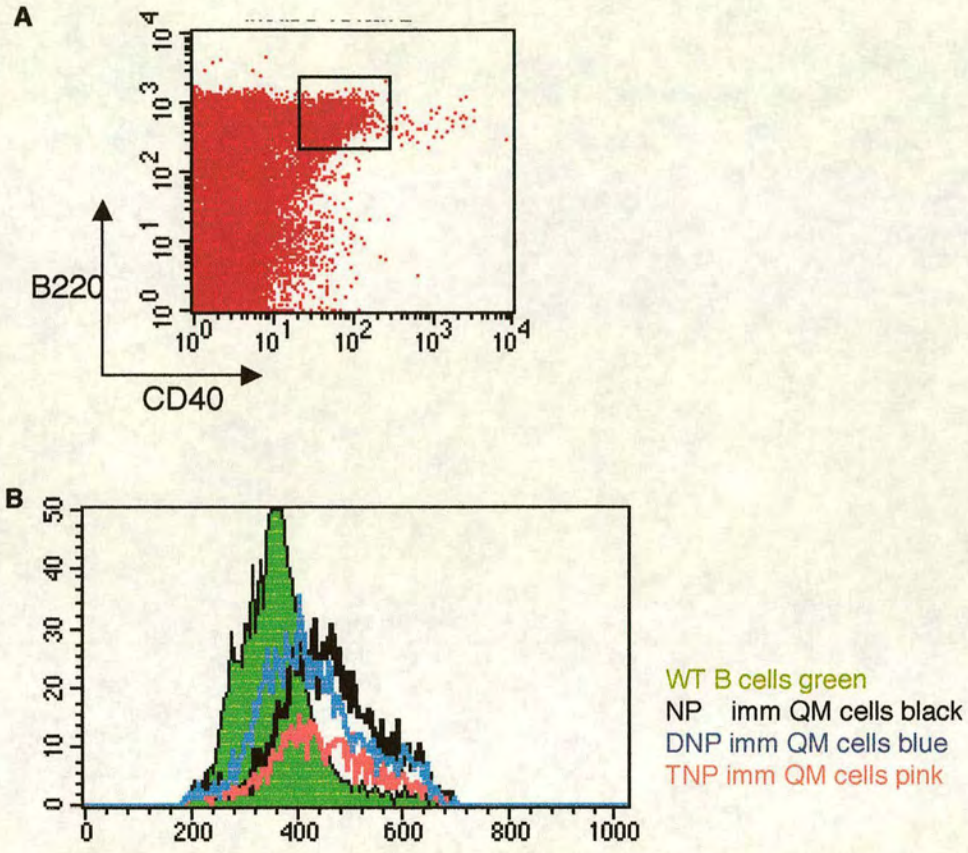
In mice that did not receive transferred cells, $CD40^+$ B cell levels are not above background. In those which received negative cells to account for contamination of the positive population, only mice immunised with NP have $CD40^+$ cells. This is due to the fact that the negative sorted population is contaminated with 1% positive cells and there is a high level of selection for and expansion of $CD40^+$ cells in these mice. In the mice that received positive cells, $CD40^+$ B cells can be detected, indicating their survival to day 21. They have, however, expanded in the NP immunised mouse but not in the DNP or TNP immunised mice.

Forward scatter profiles of transferred cells were analysed to assess their activation status, by gating on $CD40^+$ cells. Transferred cells in all mice were larger than $CD40^+$ B cells in a wt mouse, however, in mice immunised with NP cells were larger than those immunised with either TNP or DNP (Figure 4.10) indicating antigenic activation.

4.9 Germinal centres were formed in mice that received QM Id^+ cells.

Spleen sections were cut and examined for GCs. Sections were stained with PNA and surface Id with anti QM antibody to reveal whether cells in the GC had originated from transferred QM Id^+ cells.

Figure 4.10 Forward scatter analysis of transferred cells



Splenocytes of chimaeric mice that received QM^+ sorted cells were stained for B220 and CD40. Gating on transferred, $CD40^+$ B cells forward scatter profiles were compared between mice immunised with NP, DNP or TNP (**B,**black,blue and pink respectively). WT $CD40^+$ B cell were used to assess a normal forward scatter profile (green)
Profiles shown are from one mouse for each antigen and are representative of 3 - 5 mice per group.

Mice that received QM Id^+ cells and were immunised with NP formed many large GCs, most of which also stained for QM Id (Table 4.2). 3 out of 4 control mice that received sorted QM $^-$ cells also had GC but at much lower numbers. This is probably due to the very low numbers of QM Id^+ cells that are present in the QM Id $^-$ population. This again indicates that a small number of cells can generate an immune response in this system, as we estimate that these mice receive only ~100 QM Id^+ cells.

Mice that received QM Id^+ cells, but were immunised with DNP or TNP also had some GC (Table 4.1). All of the GC in DNP and TNP immunised mice were QM Id $^-$. None of the mice that received QM Id $^-$ cells and were immunised with other antigens had any GC implicating QM Id $^+$ cells in GC formation. None of the mice (5/5) that were immunised in the absence of cells, had GC indicating that any formed were from transferred cells.
GC numbers are shown in Table 4.1.

Table 4.2 GC numbers in chimaeras that received QM B cells

Antigen	QM Id positive cells	QM Id negative cells	No cells
NP	34 (34) 17 (17) 38 (30)	1 (1) 2 (2) 9 (9) 0	0 0 0
DNP	0 3 (0) 3 (0)	0 0 0	0 0
TNP	9 (0) 1 (0) 0 0	0 0 0 0	0

GCs in chimaeras that received QM positive, negative or no cells and were immunised with NP, DNP or TNP. QM Id positive GC numbers are in brackets.

4.10 Immature cells from the bone marrow do not respond well to other antigens.

The source of RAG expressing B cells has been attributed to immature cells emerging from the bone marrow (143, 147, 149). To ascertain whether these immature cells can revise their receptors in response to different antigens, we transferred bone marrow cells from QM mice to chimaeric hosts. Mice received $6x10^6$ bone marrow cells, which had been depleted of mature T cells (to avoid graft versus host disease), and were immunised 24hr later with NP, TNP or DNP. A very strong NP IgG response was generated on day 7 and was

maintained to day 21 (Figure 4.11). A very small but reproducible DNP response was detected on day 21 but there was no response to TNP.

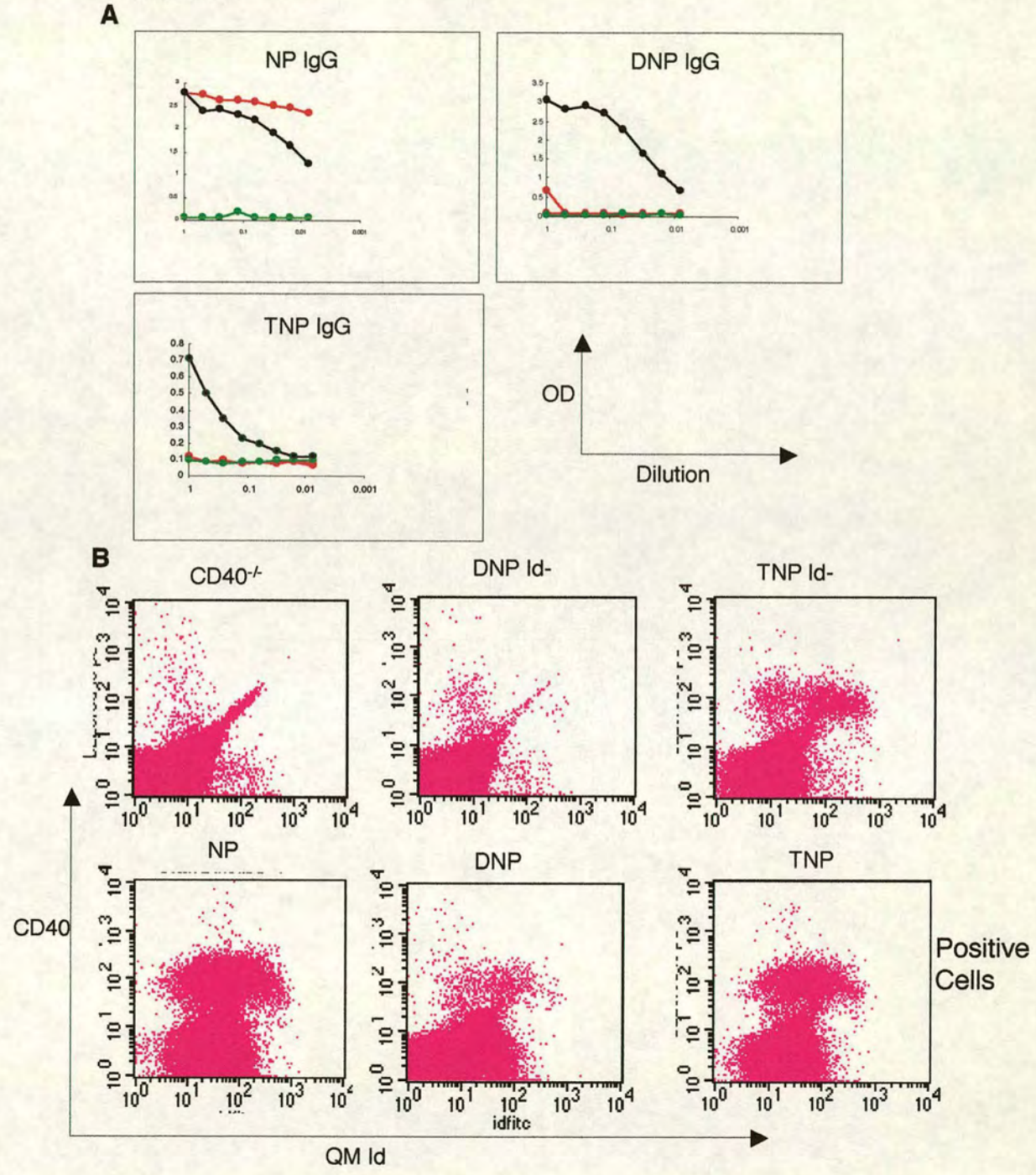
Transferred $CD40^+$ cells can be detected by FACS, however, due to the problems detailed in section 4.7, it is difficult to tell whether they are Id positive or not (Figure 4.11). Spleen sections were also analysed for the presence of GC, results are shown in Table 4.2. The mouse that generated the DNP response had higher numbers of $CD40^+$ B cells and also had a large number of GC, only half of which are Id positive (Table 4.3).

Table 4.3 GC numbers in chimaeras that received QM bone marrow cells.

Antigen	Number of GC
NP	28 (26)
DNP	28 (11) 0 (0)
TNP	15 (14) 5 (3)

GC numbers in chimaeras that received $6x10^6$ QM bone marrow cells. Numbers in brackets indicate the number of QM Id^+ GC.

Figure 4.11 Response of transferred bone marrow cells to NP, DNP and TNP



6x10^6 QM bone marrow cells were transferred to chimaeras and mice immunised with NP, DNP or TNP. Mice were bled on day 21 and IgG ELISAS performed **A** Antigen specific ELISAs: experimental mice are in red with positive and negative controls in black and green respectively. **B** Spleens were harvested and stained for B220, CD40 and QM Id. Plots shown are gated on lymphocytes and B220 ^{hi} cells to avoid CD40$^+$ DC and show CD40 and QM Id expression.

Discussion

Background

Affinity maturation of antibodies is currently ascribed to somatic hypermutation. Receptor revision, the process whereby the entire variable region of an antibody is changed by RAG mediated recombination, might provide an additional means of diversification. To investigate this we needed to follow the response of a cohort of B cells that had a known specificity, so that if this specificity was altered it could be detected. We decided to use QM B cells, which are specific for the hapten NP. Some of the B cells in this mouse already recognise antigens other than NP due to central editing of their BCRs. To obtain a pure population of B cells, the NP specific cells were separated from the others by sorting them on the basis of expression of the site directed transgenic heavy chain, which provides the NP specificity. These cells were then transferred into chimaeric hosts, which lack CD40 on B cells, allowing the selective expansion of transferred cells as they are the only ones that can receive signals from T cells through CD40.

The discovery of RAG expression and DNA excision products, indicative of functional protein, in the periphery, raised the question of its function there (130, 133). RAG is required for the rearrangement of antibody gene segments to enable antibody production and was therefore thought to be active solely in the bone marrow during B cell development. Since its discovery in the periphery, there has been much speculation about the function of RAG here. It appears risky to generate random new B cells specificities, some of which could by

chance be autoreactive. Indeed, initially it was speculated by Nemazee and co workers that peripheral rearrangement was a tolerance mechanism just as receptor editing is in the bone marrow. This turned out not to be the case as RAG expression is turned off on the receipt of a strong signal through the BCR (137). This result was corroborated in human tonsillar B cells (139). These results along with the sequencing of human B lymphocytes revealing 'hybrid' VH gene segments allayed fears that the phenomenon was a transgenic artefact (155).

We and others have suggested that receptor revision might play a role in providing useful specificities in the GC, which would be unobtainable by somatic mutation alone (133, 157, 175). Our working hypothesis is that receptor revision and somatic hypermutation play complementary roles in affinity maturation. Somatic mutation might have limited effects on certain antigen binding structures with further mutation resulting in loss, rather than gain, of binding affinity.

When this work was started no evidence of a role for receptor revision had been documented. Research focussed on the investigation of the nature of RAG expressing cells with a number of reports suggesting that the cells were, in fact, immature immigrants from the bone marrow (143, 149). Immature cells express RAG to make functional antibody, if cells were to leave the bone marrow while still expressing RAG they might be mistaken for mature cells in the GC as they share some cell surface markers (GL7). This data is difficult to reconcile with PCR and immunohistochemical evidence of RAG expression in lymph nodes where bone marrow cells are unlikely to migrate (131, 132). *In vitro* experiments also directly demonstrate receptor revision in transgenic B

cells of splenic origin upon stimulation only in the presence of low affinity antigen (137).

Irrespective of the source of RAG expressing cells we sought to find out whether they could contribute to affinity maturation of the immune response. Recently some publications have addressed the role of peripheral revision. Goossens and co workers (156) state that

> "Receptor revision plays no major role in the shaping of the receptor repertoire of human memory B cells after the onset of somatic hypermutation."

They analysed $V_\kappa J_\kappa$ and $V_\lambda J_\lambda$ joints in single λ positive GC B cells for rearrangements at the κ and λ loci and found that the frequency of rearranged cells is less than 3%. However, Magari and co workers (176) analysed the production of λ positive anti pNP IgG antibodies and say that their findings suggest that

> "Light chain rearrangement that occurs in the periphery can contribute to affinity maturation of antibody."

We felt that the issue was as yet unresolved and used a novel system to investigate further.

Development of a system to allow the distinction of the antibody response of transferred cells.

Having developed an adoptive transfer system, which we felt provided a physiological environment for the receipt of sorted cells, we wanted to ensure that the system specifically allowed proliferation of a cohort of identical transgenic cells. Previous experiments investigating the source of RAG

expressing cells used empty vessel RAG deficient hosts (143, 147, 149). The ability of cells to divide in response to antigen was not investigated in all of these studies. We showed by CFSE labelling cells that when immunised with NP they undergo numerous rounds of cell division, confirming the suitability of our host.

Secondly, to determine whether a minor population of cells might be able to provide a serum antibody response unsorted QM splenocytes (about 25% of which have diverse specificities) were transferred. This was to determine whether if a small proportion of the transferred pure QM positive cell population were to alter specificity and recognise another antigen, we might be able to detect the response by ELISA. The responses to RCF and DNP detected along with the increase in QM Id $^-$ cells encouraged us that we might be able to detect a response from revised cells.

Another way to do this might have been to spike sorted cells with transgenic cells of another specificity. This would have given us an idea of how many cells are required to give a detectable immune response. However, our results where QM Id $^-$ sorted cells which contain ~1% QM Id$^+$ cells give a detectable ELISA response on day 14 and 21 indicate that the response of a very small number of cells can be detected in this system. We estimate that about 100 QM positive cells were transferred in this case. In early control experiments where C57BL/6 B cells were transferred to chimaeric hosts, responses were detected in mice that received as few as 1x10^6 cells. Mice were immunised with NP-OVA and although the number of NP specific cell must have been very low, an NP specific antibody response was still detected. In experiments to analyse

the response of QM positive cells to non-NP antigens between 2 and 3 x10^6 QM cells were transferred, all of these cells would have had the opportunity to interact with TNP and DNP. If receptor revision was to be induced by one of our low affinity antigens and be physiologically relevant we should have been able to detect it as we had a greatly increased number of relevant B cells.

Our main concern when devising an experimental strategy to detect receptor revision was that sorting was never going to provide as pure a population of B cells as we would want. Contamination with B cells of other specificities was minimised to 1%, this required extensive optimisation of the sorting conditions. To control for this contamination the experiment was set up with an equal bias for mice receiving sorted positive and negative cells. Also another control group of mice, which did not receive cells, was included to allay any fears of a serum IgG contribution from 129xB6 F1mice, the irradiated hosts for the generation of bone marrow chimaeras. In the event it might have been more beneficial to bias the experiment more toward the mice that received positive cells as most of them made no response to DNP or TNP and our fears of the contribution of negative cells may have been exaggerated.

Antibody responses attributable to transferred cells.

A strong NP specific IgG response was detected in mice that received transferred cells and were NP– OVA immunised, indicating that cells transferred to chimaeras can make good antibody responses. This contrasts with lower levels of antigen specific Ig secreted by transgenic cells transferred to normal hosts by other groups (191, 192). Initially, most of the antibody produced by transferred cells was of the isotypes IgG2a and 2b, probably due to

the use of *Bordetella pertussis* , which is a Th1 inducing adjuvant. With time, however, the nature of the response changed and by day 21 IgG1 was the main contributing isotype. The mice that received sorted QM Id $^{-}$ cells responded to NP but not to DNP or TNP, leading us to conclude that the NP response was caused by a small number of QM $^{+}$ cells, present due to sorting inaccuracies. Due to the lack of response to DNP and TNP in mice that received negative sorted cells we believe that the small number of QM negative cells present in the QM positive population is not responsible for the TNP response detected on day 21.

Based on preliminary findings we had expected not to see any response to either DNP or TNP from sorted cells. The fact that a small TNP response was detected on day 21 in 2 mice that received QM Id^{+} cells but not in those that received QM $^{-}$ cells is intriguing. However, the fact that a response is detected in 2 mice in one experiment and not in another 2 mice in a different experiment does not allow an unequivocal conclusion to be drawn.

One possibility is that the response to TNP is caused by QM positive cells, which having revised their receptors, are now Id negative. However, somatically mutated QM positive cells, or QM negative cells already present in the transferred population could also be responsible. The fact that mice that received negative cells did not provide a TNP response implies that it is derived from QM positive cells leaving either revision or mutation as possible causes. This issue could have been resolved if we had been able to cross QM mice onto a RAG deficient background. Crossing to RAG deficient mice rules out a

contribution from receptor revision as RAG is required for receptor rearrangement. However, this cannot be carried out as QM B cells require RAG expression to rearrange endogenous lambda light chains to make a functional antibody. If we had still obtained a response to TNP in B cells deficient in RAG we could have attributed it to somatic mutation alone.

We might have expected that affinity maturation by somatic hypermutation would have given us some response to the chosen antigens. Most of the work on somatic mutation has focussed on the molecular diversity generated. Those assessing affinity changes have generally generated hybridomas and assessed affinity by fluorescence quenching (158, 159). Affinity maturation of serum antibodies, however, has been described by Smith and co workers (193), and can cause a 1000 fold increase in affinity to antigen. Indeed somatic mutation can cause a complete change in specificity between haptens (194) or result in loss of binding (195). These facts need to be borne in mind when drawing conclusions from our data.

Due to the complex nature of these experiments we chose a small selection of antigens against which to measure affinity maturation. In the 3.83 system, which formed the basis for our experiment, receptor revision was triggered by low affinity antigen. This affinity did not induce proliferation of transgenic B cells nor did it increase their forward scatter (FSC) profile as analysed by FACS (201). The forward scatter profiles of QM cells transferred to chimaeras were analysed. Cells from mice immunised with NP had a larger FSC profile that those from mice immunised with either DNP or TNP. However, all transferred cells were larger than B cells from a C57BL/6 mouse. This may reflect the fact that these cells had been adoptively transferred and

were the only $CD40^+$ B cells in the mouse, or it may truly effect antigen activation.

All that was required, to trigger V(D)J rearrangement in the 3.83 system was receptor occupancy as measured by mean fluorescent intensity of samples analysed by FACS. We were not able to assess receptor occupancy of QM receptors with DNP or TNP due to a lack of the required reagents. In this respect the 3.83 system is better defined. Our results obtained from the 3.83 system are described in chapter 5, however, the nature of the antigen caused unforeseen complications, justifying our parallel investigation of receptor revision in QM mice with the better-defined hapten-carrier system.

Analysis of germinal centre formation by transferred cells

Immunofluorescent analysis of spleen sections revealed that GC were formed in mice that received QM positive cells only. Mice that did not receive cells or those that received negative cells did not have GC (apart from those that were immunised with NP). This implies that the GC observed originate from transferred QM positive cells. In mice that were immunised with NP most of the GC detected were QM Id^+, however, a small number were Id negative. The GC present in NP immunised mice that received negative cells are also QM Id^+ indicating that they were probably formed by contaminating QM $^+$ cells in the negative population. We do not know why some of the GC did not express Id but it may indicate an unreliability of the anti QM Id antibody on sections.

It is a possibility that the QM staining observed is not actually expression of QM Id on the surface of GC B cells. GC B cells are known to down regulate

surface Ig so in fact we may be detecting the binding of serum antibody to Fc receptors on B cells. It is unlikely that we are observing the binding of antibody to FDC networks as the staining does not have the characteristic branching appearance detected when immune complexes on FDC networks are stained with anti IgM. Fc receptor mediated binding may explain why only NP immunised mice have QM positive GC. NP immunised mice that received wt cells are Id negative indicating that this phenomenon is not only related to NP immunisation but may be due to the abundance if Id positive serum present in this system. If this is the case it would be analogous to the situation detected when performing FACS analysis on the spleens of these mice.

Mice that were immunised with TNP and DNP had low numbers of GC, all of which were QM Id $^{-}$. Since these mice received sorted QM positive cells we might have expected the GC to be QM positive if those cells had been recruited to GC by low affinity interactions with antigen. It is known that low affinity B cells can form GC in the absence of competition (196, 197). However, the GC in DNP and TNP immunised mice are not QM positive.

One explanation is that in mice immunised with TNP and DNP there is selection for B cells of other specificities to form GC. These may be the negative cells in the positive population, however, mice that received negative cells only, do not have GC implying that they were formed by QM positive cells. They may also be positive cells that have lost Id expression either due to secondary rearrangement or somatic mutation, we are unable to tell using this system.

FACS analysis of transferred cells

Despite the fact that QM mice cannot be crossed onto a RAG deficient background allowing a comparison of the response in mice that can and cannot undergo receptor revision, we had hoped that analysis of Id expression by FACS would reveal whether cells had lost expression of the idiotypic receptor. This alone may have elucidated whether of the response to TNP had come from either QM positive or negative cells. Having transferred sorted QM Id^+ cells, these would be the only ones detectible by FACS in the absence of secondary V gene rearrangement. However, if cells underwent receptor revision and lost specificity to NP we should be able to detect this by enumerating $CD40^+$ cells which no longer expressed the QM Id.

As mentioned in the results section, several problems were encountered with FACS staining of splenocytes from experimental mice. Firstly $CD40^-$ B cells of the host, which should be QM negative, appear to be bound by the anti Id antibody making it difficult to distinguish truly positive cells. Secondly transferred $CD40^+$ QM Id^+ B cells appear less brightly stained for QM Id than QM cells do in a QM mouse. We attribute this to activation induced down regulation of surface Id as most of the cells have been shown to divide in response to immunisation with NP (section 3.11). Thirdly, autofluorescent cells in the spleen, which lie in the same position as transferred cells in the dot plot make quantitative analysis untenable. These issues are discussed in more detail below.

The staining pattern observed using the anti QM Id antibody is neither due to antibody batch nor the genetic background of the mice employed as both of these options were investigated. When comparing mice, only those that received QM Id^+ cells have CD40 deficient cells that are bound by anti QM Id antibody. CD40 deficient cells in NP immunised chimaeras that did not receive QM cells do not bind QM Id. This indicates that the phenomenon is related to transferred QM Id^+ cells. We propose that the high levels of QM Id antibody secreted by transferred cells is bound by Fc receptors on B cells (T cells are not shifted), this is then detected by the anti Id antibody when staining cells *ex vivo*. In fact, when examining a QM mouse the same phenomenon can be observed, QM Id^- cells are shifted strongly to the right, however, as the positive cells are not activated they can be distinguished as they are brighter still.

In previous experiments transferred cells could easily be detected at day 7 or 14. However, in this experiment mice were repeatedly boosted resulting in very high NP specific serum IgG. This antigen specific serum is also of a single Id arising from transferred cells only. These factors may explain the binding of QM Id to CD40 deficient host cells in chimaeras that received transferred QM cells.

Secondly, although $CD40^+$ transferred QM cells in the spleen are not stained much more brightly than host cells as mentioned before we believe this may be due to the down regulation of sIg when cells are activated. We do not think it possible that all transferred cells have lost Id expression, which is another explanation for the profiles observed. All mice that received QM cells

and were NP immunised have high levels of QM Id $^{+}$ GC implying that there are QM Id $^{+}$ cells in the spleen. Also when examining Id staining by FACS the stain is continuous implying that there is just one population - that of positive cells. There is no reason for NP immunised QM cells to alter specificity by receptor revision as they bind the antigen with high affinity and the receipt of a strong signal through the BCR has been shown to down regulate RAG expression. The diminution of Id staining on QM Id^{+} cells means that we cannot clearly distinguish QM Id^{+} cells from those of the host on the basis of QM Id expression.

We now realise that our aim to visualise transferred cells that may have revised their receptors by FACS was ambitious. Even in the absence of the problems encountered and discussed above the detection of a small number of cells that may have undergone revision may not have been possible. However if cells were to revise in response to antigen we might expect them to be selected and to expand, improving their chances of detection.

Transfers of QM bone marrow cells to adoptive hosts

To address directly the issue that bone marrow cells, expressing RAG are the cells that were detected in the spleen (143, 147) we investigated whether they could contribute to affinity maturation. Very strong NP responses were detected indicating that bone marrow cells can mount a response however, responses to other antigens were absent or very low. A small response, to DNP was detected on day 21. This implies that cells from the bone marrow, although they may indeed be the cells reported by others in the spleen do not make a

significant response to antigens other than those they are already specific for. This may indicate that immigrant cells from the bone marrow cells cannot revise their receptors to any considerable level.

Conclusions

In retrospect, aspects of this experiment were perhaps too ambitious and needed to be refined more fully. Due to the delays incurred in the generation of a suitable adoptive transfer system a more basic analysis of the affinity maturation or responses was necessary. The initial experimental plan involved the generation hybridomas by fusion of the spleens of experimental mice. This would have afforded the opportunity to gain unambiguous information about the affinity of individual clones by using surface plasmon resonance (198, 199). Sequencing of clones would have elucidated the make up of the V region and given information about the contribution of somatic hypermutation and receptor revision. Further analysis of the affinity of the BCR for the selected antigens would also have been beneficial this however, was impossible due to time restrictions.

We have however, developed a novel adoptive transfer system that has been demonstrated to improve the strength of the response of adoptively transferred cells. We have shown that a cohort of transferred cells can make antibodies and form GCs in response to antigens other than that recognised by the site-directed transgenic BCR they possess. One explanation for these results is that cells have undergone receptor revision of their BCRs, however, we cannot rule out that they may also have undergone somatic hypermutation alone. The

formation of unequivocal conclusions from these results is difficult and we would be reticent to do so in the absence of further investigation.

Chapter 5 Receptor revision in 3.83 B cells

Introduction

In splenic B cells in the 3.83 transgenic mouse, V(D)J recombination has been shown to be inhibited upon the receipt of a defined strong signal through the BCR, but not by a weak signal (137). This was interpreted to mean that B cells with high affinity for antigen, having no reason to alter specificity, continue to express the same BCR expression. Whereas B cells that only bind the antigen weakly are given the opportunity to bind it more strongly by receptor rearrangement.

The 3.83 BCR used in this experiment was selected as source of transgenic B cells for experiments described in this chapter. This allows the investigation of affinity maturation in a B cell population known to undergo secondary rearrangements in the periphery, following the receipt of a defined weak signal. However, instead of using the 3.83 transgenic mouse as previously employed, we used the 3.83 knock-in mouse (3.83 KI) (177, 200) (19), kindly donated to us by Dr Roberta Pelanda (Denver, Colorado). In this mouse the pre-rearranged 3.83H and 3.83κ genes were targeted to the endogenous loci resulting in the generation of a mouse in which ~90% of the B cells have the same specificity. These B cells are also able to undergo somatic hypermutation and class switching of the inserted Ig genes, unlike those of conventional transgenic mice where the transgene is randomly inserted.

The ligand for the 3.83 BCR is the MHC class I molecule $H\text{-}2K^{K}$, it also recognises $H\text{-}2K^{b}$ with lower affinity, while binding to $H\text{-}2K^{d}$ is below detection. To facilitate antigen generation, a recombinant phage library

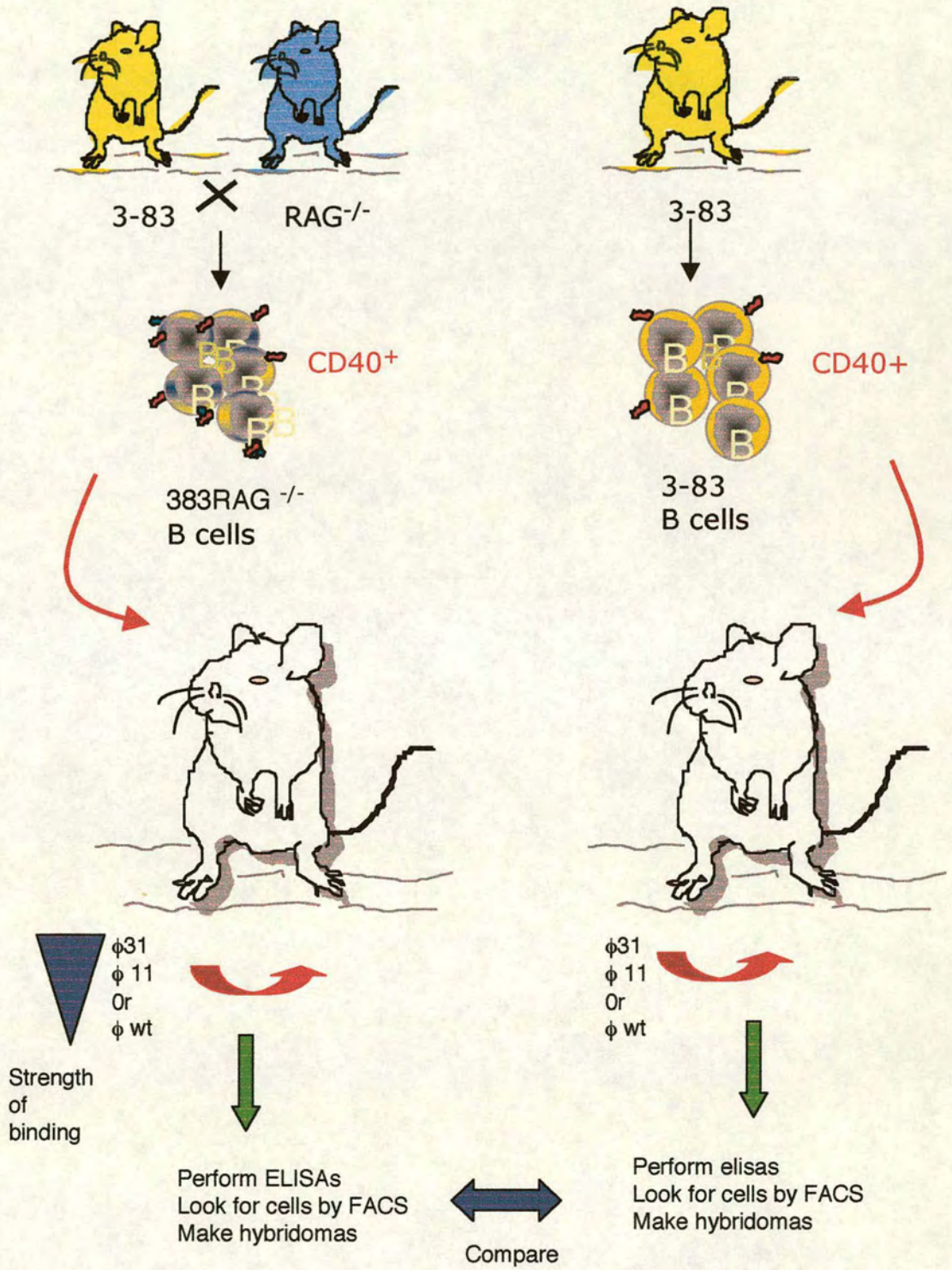
was screened for clones recognised by the BCR with the above hierarchy. Three phage known as ϕ31, ϕ 11 and ϕ wt, mimic the high, low, non binding properties of the MHC molecules (201). It is in response to ϕ11, but not to ϕ31 or ϕwt, that receptor revision was detected in 3.83 transgenic mice (137). We adopted this antigen system to investigate if secondary rearrangements in response to immunisation with ϕ 11, might allow affinity maturation of the B cell response to this antigen.

In addition, to evaluate the contribution of receptor revision alone to affinity maturation, we wanted to compare the response of pure B cells from RAG sufficient mice with that of RAG deficient B cells, which are unable to rearrange antibody genes. 3.83 KI mice were bred with RAG 2 deficient mice resulting in a mouse in which the only lymphocytes are B cells expressing the pre-rearranged 3.83 BCR. These B cells are unable to undergo further receptor rearrangement, however in the presence of T cells should be able to somatically hypermutate. These cells were used as a comparison with RAG sufficient cells in adoptive transfer experiments. This allows a distinction to be made between the contributions of receptor revision and somatic hypermutation to affinity maturation by comparing the affinity of serum from cells which can both mutate and revise with those that can only undergo mutation.

Having developed an adoptive transfer system for the QM mice that allowed detection of antibody responses on the basis of IgG production, we decided to adopt an analogous approach for the 3.83 mice. B cells from 3.83 KI mice were sorted on the basis of 3.83 expression to provide a pure population. These cells were transferred to chimaeric hosts and mice immunised with one of the high affinity, low affinity or control, non-binding phage. In parallel, mice

received 3.83 RAG cells. IgG responses were determined by ELISA as measure of the response of transferred cells (Figure 5.1). Results obtained from this system are presented in this chapter.

Figure 5.1 Schematic diagram of 3.83 experimental system



Results

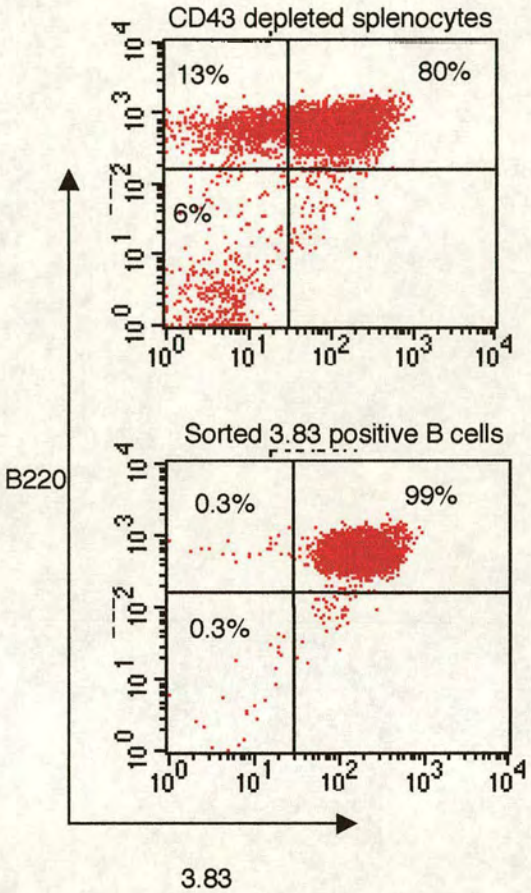
5.1 3.83 mice contain centrally edited cells that can be removed by cell sorting.

Unlike QM mice, 3.83 mice have normal levels of B cells; this makes isolation of those that express the transgenic receptor cells less difficult. They also have lower levels of centrally edited cells, only about 10% of B cells have lost expression of the transgenic receptor. B cells that express the 3.83 BCR can be detected with the anti idiotypic antibody 54.1 (15), allowing their separation from B cells of different specificities. Splenocytes were harvested from 3.83 mice and enriched for B cells by CD43 depletion of other cells types. Cells were then stainedwith antibodies to B220 and 3.83 Id. Double positive and single positive cells were isolated using a FACS Vantage cell sorter. Typically 5% of the starting splenocyte culture was yielded as double positive B cells, this contrasts with only 1% for QM mice. Sort purity was also improved with between 0.2 and 0.4% Id negative B cells present (Figure 5.2).

5.2 Centrally edited 3.83 cells can respond to some antigens.

When QM mice were immunised with a selection of model antigens they were able to make a response to all of those tested. 3.83 mice have a less diverse repertoire, 10% as opposed to 25% of their B cells express a non-transgenic receptor. To ascertain whether these cells could mount a serum antibody response to a selection of antigens, mice were immunised with NP-OVA, Ox-CSA and RCF.

Figure 5.2 3.83 sort purity.



3.83 splenocytes were depleted of non B cells by CD43 MACS. 3.83 positive cells were sorted on the basis of binding B220 and 54.1 (anti 3.83 Id) using a FACS Vantage flow cytometer. To assess sort purity 3.83 positive and negative populations were analysed on a FACS Calibre flow cytometer. Shown above are representive examples of samples after CD43 depletion (top) and after cell sorting (bottom)

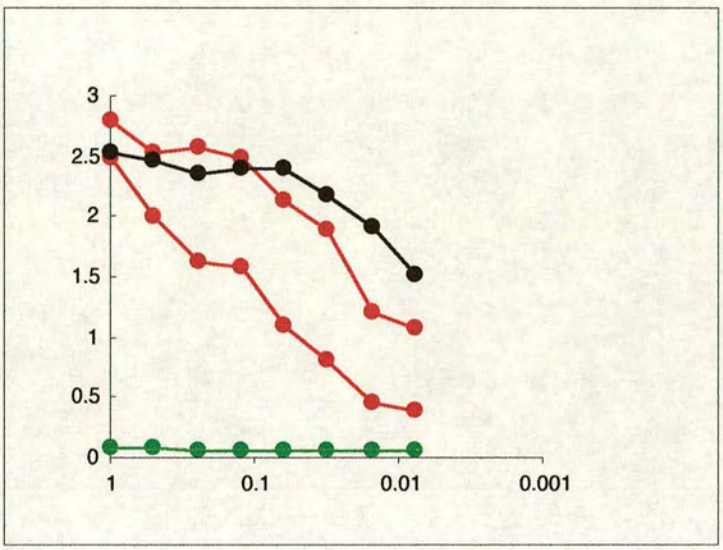
Mice failed to respond to either hapten but both 3.83 mice immunised made responses to RCF (Figure 5.3). This indicates that a small number of centrally edited cells can provide a serum antibody response and reinforces the need to remove cells of non-transgenic specificity in order to examine responses only those cells that express the transgenic Id. The failure to respond to either hapten may be related to the greatly reduced diversity of B cells in the repertoire of these mice.

5.3 3.83 mice crossed to a RAG deficient background contain B cells of a single specificity

Unlike QM B cells, which require RAG expression to generate functional antibody. 3.83 KI mice can be crossed to RAG deficient mice as they carry insertions of both heavy and light chain antibody genes. 3.83 KI mice were mated with BALB/c RAG 2 $^{-/-}$ mice to produce mice homozygous for both heavy and light chain genes and the disrupted RAG gene. To examine the make up of cells in the spleen, splenocytes from 3.83 RAG mice were stained for 3.83 expression with anti 3.83 Id (54.1 (15)), B cells and T cells were stained with monoclonal antibodies to B220 and CD3 respectively.

The RAG-deficient 3.83 KI mice have no mature T cells due to their inability to rearrange T cell receptor genes. All B cells are Id positive (data not shown), unlike 3.83 KI mice in which about 10% of the B cells are Id negative.

Figure 5.3 Response of 3.83 mice to immunisation with RCF.



3.83 mice were immunised with 100μg of alum precipitated RCF and boosted 10 days later. On day 14 blood samples were taken and serum antibody responses analysed by ELISA. IgG responses to RCF of two immunised 3.83 mice are shown above in red. The response of a BALB/c mouse is depicted in black and the negative control is depicted in green.

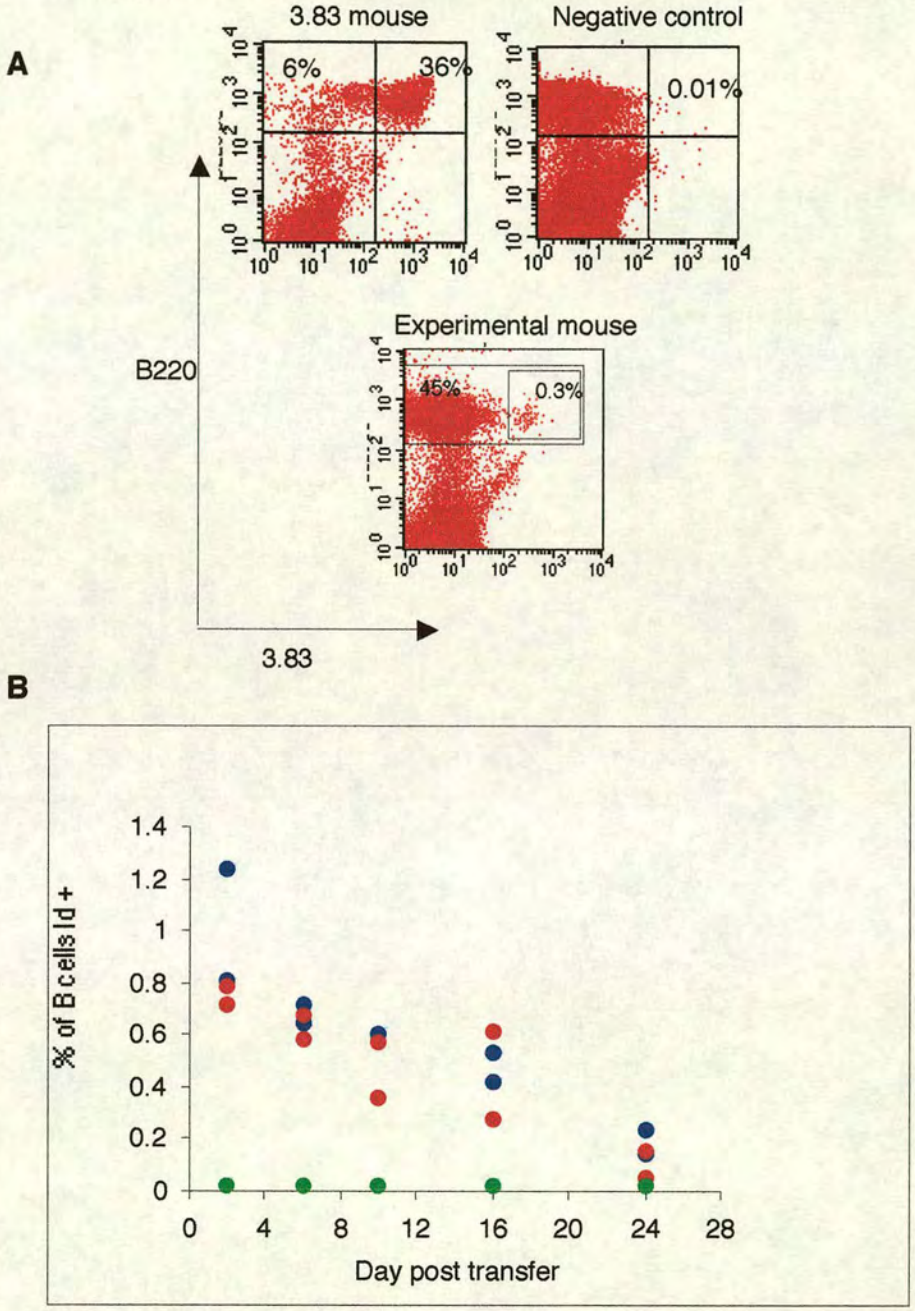
5.4 Survival of 3.83 cells, transferred to BALB/c mice .

To confirm that 3.83 cells could survive transfer to mice on a BALB/c background, $1x10^7$ 3.83 splenocytes were injected i.v. into BALB/c mice and immunised 24hr later. Immunisation for the 3.83 system is performed subcutaneously (s.c.) in the hind legs with 50μg of phage emulsified in complete Freund's adjuvant (CFA). On day 2,6,10,16 and 24 post transfer 2 mice were sacrificed and spleens and draining lymph nodes examined for the presence of transferred cells by FACS. Cell numbers slowly decline from a maximum of about 0.8 % of B cells on day 2 to 0.2 % on day 24 (Figure 5.4), indicating that cell stably survive adoptive transfer. Equal ratios of positive cells were found in the lymph nodes and the spleen.

5.5 Analysis of recombinant M13 Phage, ligands for the 3.83 BCR.

Recombinant M13 phage, which express mimotopes recognised by the 3.83 BCR, were a gift from Dr Valerie Kouskoff (Denver, Colorado). The mimotope is expressed as a pIII fusion, this is the minor coat protein of the phage and is present in four copies per phage particle. Phage are isolated from infected bacterial cultures by polyethylene glycol (PEG) precipitation. To confirm the identity of the 3 phage types (ϕ31, ϕ11 and ϕwt, high, low, and non binding respectively) and their behaviour in our hands, we performed several tests.

Figure 5.4 3.83 B cells survive transfer to BALB/c mice.

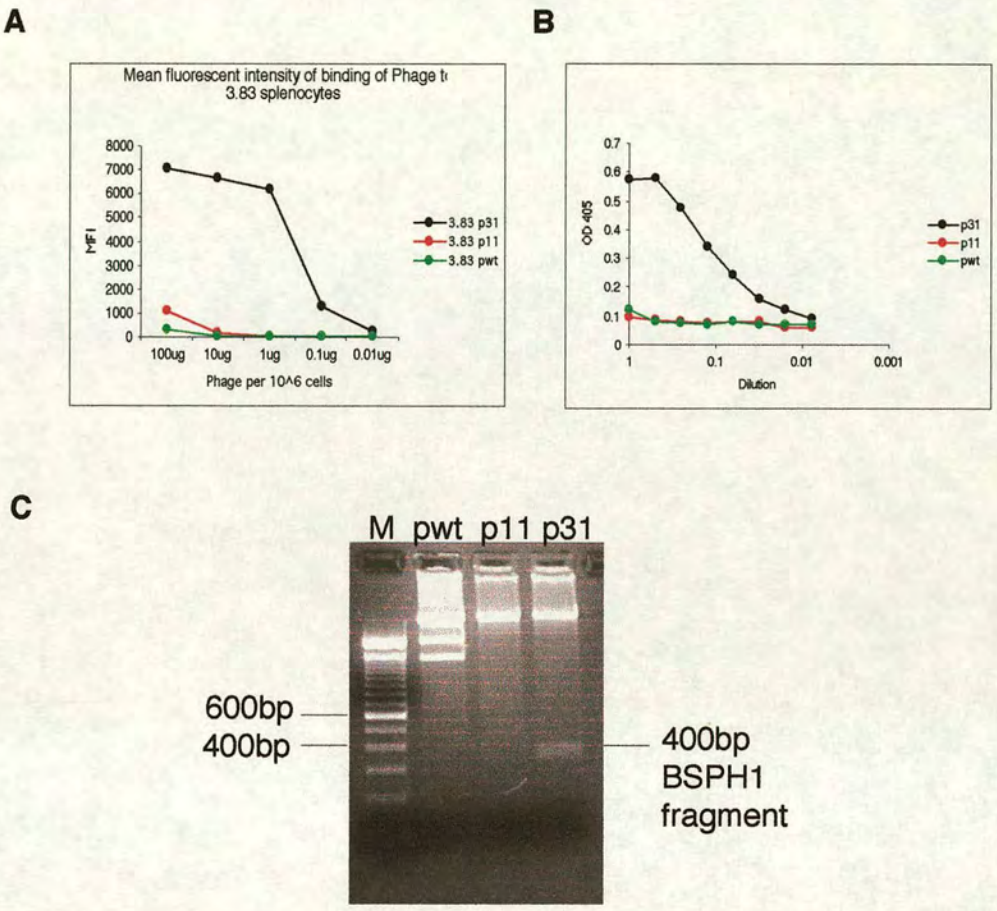


1x10^7 3.83 splenocytes were transferred to BALB/c hosts and mice were immunised 24hr later. On day 2,6,10,16 and 24,2 mice were sacrificed and spleens harvested for analysis of transferred cells. BALB/c mice that did not receive any cells were also Immunised and used as negative controls. **A** Splenocytes were stained with antibodies to B220 and the transgenic B cell receptor. The percentage of 3.83 positive cells was assessed and is depicted in **B.** The percentage of B cells that were Id positive in the lymph nodes of analysed mice is represented in blue. Spleen results are represented by red dots and immunised BALB/c mice are represented by green dots.

The binding of the 3 phage by 3.83 B cells was assessed by measuring mean fluorescent intensity by FACS. 3.83 splenocytes were incubated with 5 serial 10 fold dilutions of phage starting at 100 μg/1x10^6 3.83 cells for 20min on ice. Cells were washed and phage binding detected with rabbit anti-M13 antibody and an anti-rabbit FITC secondary. B cells were co stained with anti-B220 conjugated to PE. The mean fluorescent intensity of binding of ϕ31 to 3.83 B cells was ~7 fold greater that of ϕ11 and ϕwt (Figure 5.5). However, in published experiments (201) the low affinity binder, ϕ11 binds more strongly than we detected, so to further assess ϕ11 binding ELISAs were performed. Plates were coated with purified 3.83 antibody and phage serially diluted (1 in 2) down the plate. Phage binding was detected with rabbit anti M13 antibody and horseradish peroxidase (HRP) conjugated goat anti rabbit Ig. Similar results were obtained with a failure to detect significant ϕ11 binding by the 3.83 antibody. In all experiments the phage concentration was normalised by performing phage ELISAs (Figure 5.6). DNA digests allow the distinction of ϕ11 (low affinity) and ϕ31 (high affinity) phage by the liberation of a 400bp band from ϕ 31 following digestion of phage DNA with BSPH1, however no similar test was available for ϕ 11 (Figure 5.5).

Having investigated all available options and analysed phage from 2 different sources, we felt we had no option but to accept that the mimotope was expressed in the phage given to us and continue with our experiments.

Figure 5.5 Analysis of M13 Phage.



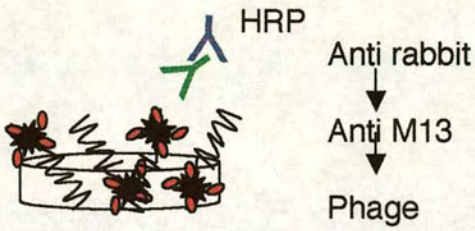
A 1x10^6 3.83 splenocytes were incubated with serially diluted phage, phage binding was revealed with ant- M13antibody and a fluorescent labelled anti-rabbit secondary. B cells were co-stained with anti B220 and analysed by FACS. Mean fluorecscent intensities of phage staining were calculated and are plotted on the Y axis against phage concentration on the X axis. ϕ31,11 and wt binding are represented by black red and green lines respectively.

B Phage binding to purified 3.83 antibody was analysed by ELISA. 3.83 was coated on plates overnight. Phage was serially diluted down plates and binding detected with anti M13 and an anti rabbit HRP secondary. ϕ31,11 and wt are represented in black red and green respectively.

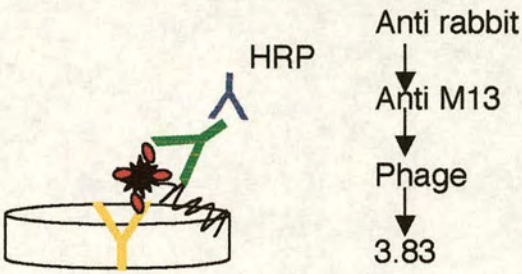
C Replicative form phage DNA was isolated from each of the 3 phage and digested overnight with BSPH1 restriction enzyme. Products were electrophoresed on a 1.2% agarose ethidium bromide gel. The 400bp liberated from ϕ31 can be detected.

Figure 5.6 Schematic diagrams illustrating phage ELISAs

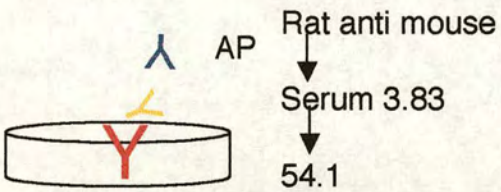
1. ELISA to detect phage



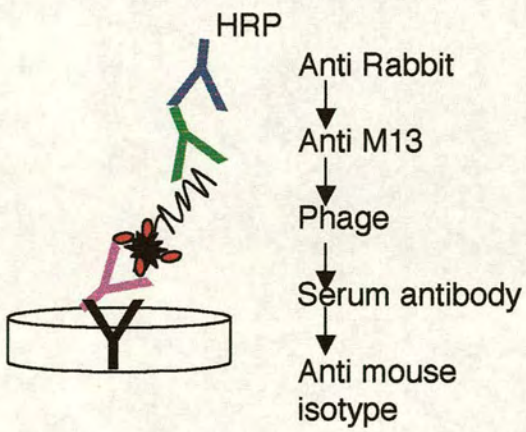
2.ELISA to detect phage binding to 3.83

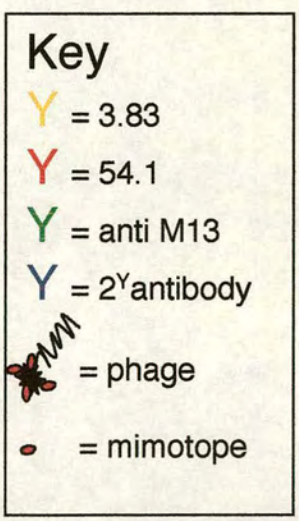


3 ELISA to detect serum 3.83 antibody



4.ELISA to detect mimotope specific serum antibody



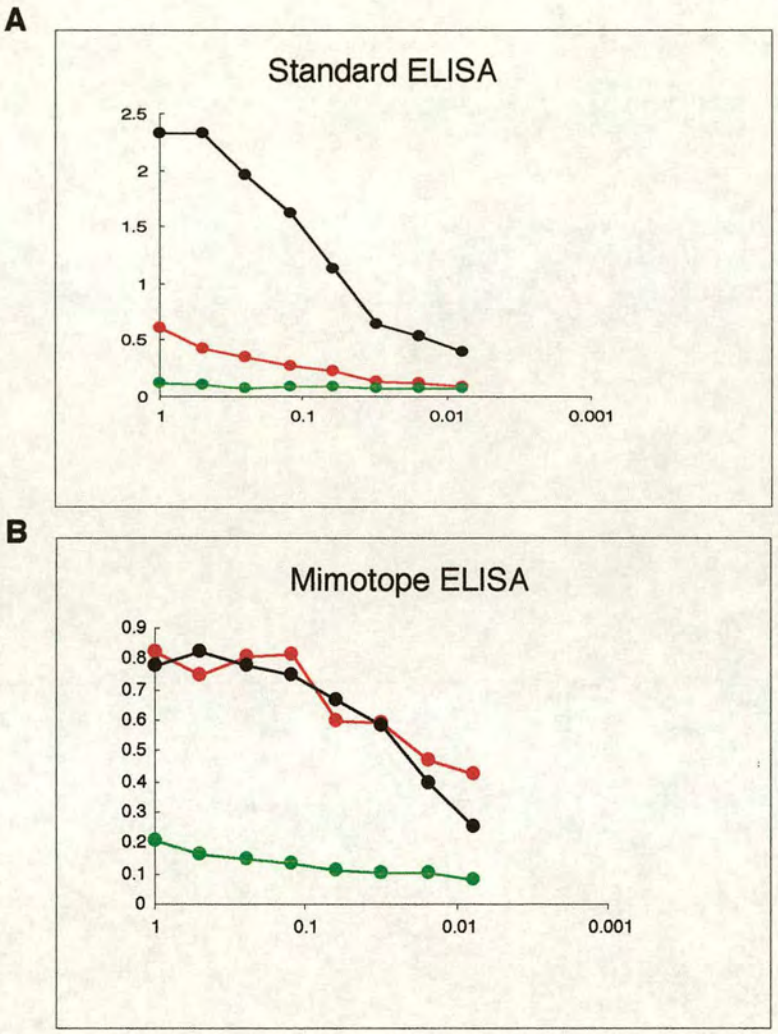


5.6 The development of mimotope ELISAs to measure serum antibody responses to phage.

Critical to our experiment is the measurement of serum antibody responses to the antigens used to immunise mice. ELISAs to measure serum antibody responses to phage mimotopes have not been previously documented. Initially a conventional ELISA protocol was adopted and phage were coated to polystyrene plates, however 3.83 mice, which have high levels of 3.83 antibody, specific for ϕ31 as detected by ELISA with anti 3.83 Id 54.1 (Figure 5.6), do not bind to phage coating ELISA plates. Phage coating plates however was bound by serum from phage immunised BALB/c mice. We recognised that the response from BALB/c mice was polyclonal, and we were measuring the BALB/c response to all of the phage epitopes combined. 3.83 antibody only recognises the mimotope, a single epitope of the phage which is repeated 4 times.

The mimotope is expressed as a pIII fusion, this is the minor coat protein and is only present in four copies per phage particle. To develop a way for the mimotope to be exposed to serum antibody, we tried lifting the phage off the plate by coating with anti M13 antibody; this did not improve 3.83 binding. However, if the ELISA is reversed, and serum placed on the bottom, bound to the plate with isotype specific antibodies, 3.83 binding to phage is possible. It emerged that the phage needs to be in fluid phase in the ELISA. This allows fixed antibody to bind the mimotope, if the phage is fixed, access to the mimotope by the antibody is hindered (Figure 5.6 and 5.7).

Figure 5.7 Mimotope ELISA sensitivity compared with standard ELISA sensitivity.



A ELISA illustrating poor binding of 3.83 serum antibody to phage coating Polystyrene plates.BALB/c (black) and 3.83(red) were immunised s.c. with phage in CFA and bled 14 days later.Serum responses to phage coating plates was measured by serially titrating serum down the plate and detecting it with anti IgG AP. Serum of a mouse immunised with an irrelevant antigen is used as a negative control (green)

B Mimotope ELISAs were performed by coating polystyrene plates with anti IgG, serum was serially titrated down the plate starting at 1 in 500. Phage was added at 0.2mg/ml to all wells and detected with ant- M13 antibody followed by an anti-rabbit HRP secondary. BALB/c, 3.83 and irrelevant serum are represented by black, red and green lines respectively.

ELISAs performed in this way are very sensitive and require very little serum. In normal serum ELISAs serial dilution starting from 1 in 50 is usual. With mimotope ELISAs the serum needs to be titrated from 1 in 500 to prevent saturation.

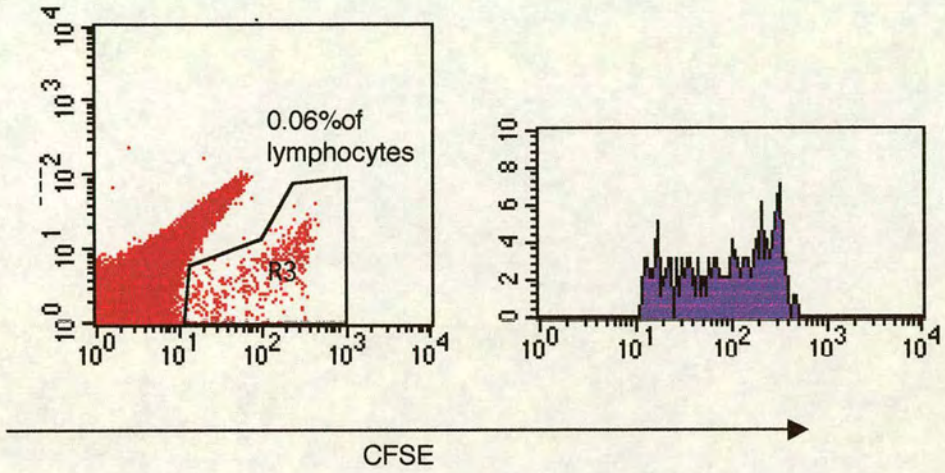
5.7 Transferred 3.83 cells divide in the chimaeric host and form germinal centres

To investigate the response of 3.83 cells to antigens of different affinity, it was decided to use the same chimaeric adoptive host system that was used for the transfer of QM cells (see chapter 4). 3.83 KI mice, however, are on a BALB/c background. We needed $CD40^{-/-}$ mice and μMT mice on the same background. Both lines were crossed to BALB/c mice for 3 generations (referred to as μMT/BALB/c or $CD40^{-/-}$ /BALB/c mice for simplicity). Chimaeras were made using either μMT/BALB/c hosts or $CD40^{-/-}$ /BALB/c hosts and mice were reconstituted as described previously with 20% $CD40^{-/-}$ bone marrow and 80% μMT bone marrow.

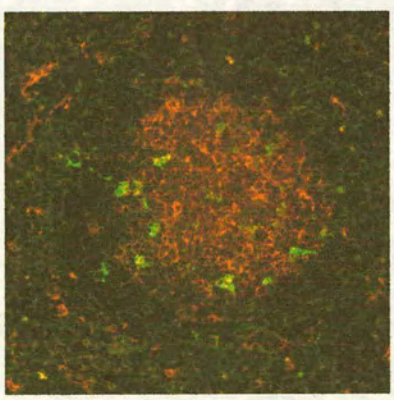
To confirm that sorted 3.83 B cells can divide when transferred to this adoptive host, sorted 3.83^{+} B cells were CFSE labelled and $1.5x10^{6}$ cells transferred chimaeric mice. Mice were immunised s.c. in the hind legs with ϕ31 emulsified in CFA 24hr later and on day 7 spleens were harvested for FACS analysis. It can be seen from Figure 5.8 that many cells have undergone 5 or more cell divisions, indicating the antigen responsiveness of transferred cells.

Figure 5.8 Cell division and germinal centre formation of transferred 3.83 cells

A



B



1.5x10^6 CFSE labelled sorted 3.83$^+$ B cells were transferred to chimaeric hosts and mice immunised s.c. with ϕ31 in CFA 24 hr later. **A** 7days later splenocytes were analysed for division of transferred cells by FACS. The mouse shown is representative of 2 analysed.
B Spleen sections were stained with PNA Texas red and anti 3.83 Id FITC to detect the presence of germinal centres, shown is one germinal centre representative of 1 of ~20 per section in 4 mice examined.

On day 17 spleens were harvested and sections analysed for GC formation by staining with fluorescently labelled PNA and anti idiotypic antibody. The anti idiotypic antibody, works poorly on sections and binds non-specifically to stromal cells making it impossible to distinguish GC from Id $^{+}$ or Id $^{-}$ cells, however, in 4 mice analysed GC numbers were the same as those in immunised BALB/c mice (18 +/- 4.2sd) (Figure 5.8). No GCs were detected in immunised chimaeras in the absence of transferred cells indicating that host cells are not responsible for GC detected.

5.8 All mice generate an IgG response to phage

A similar strategy to that described for the QM system was adopted for 3.83 cell transfers. B cells were enriched by CD43 depletion of splenocytes, 3.83 $^{+}$ cells were then sorted from Id $^{-}$ cells. Sorting 3.83 cells proved more accurate as they have more B cells and a higher percentage of them are Id positive. Purity of 99% was attained for Id positive cells, with only 0.2 to 0.4 % Id negative B cell contamination. The same emphasis was placed on controlling for sort accuracy and an equal number of mice received sorted 3.83 $^{-}$ cells as received positive cells. Control mice that received no cells were also immunised with each of the three phage.

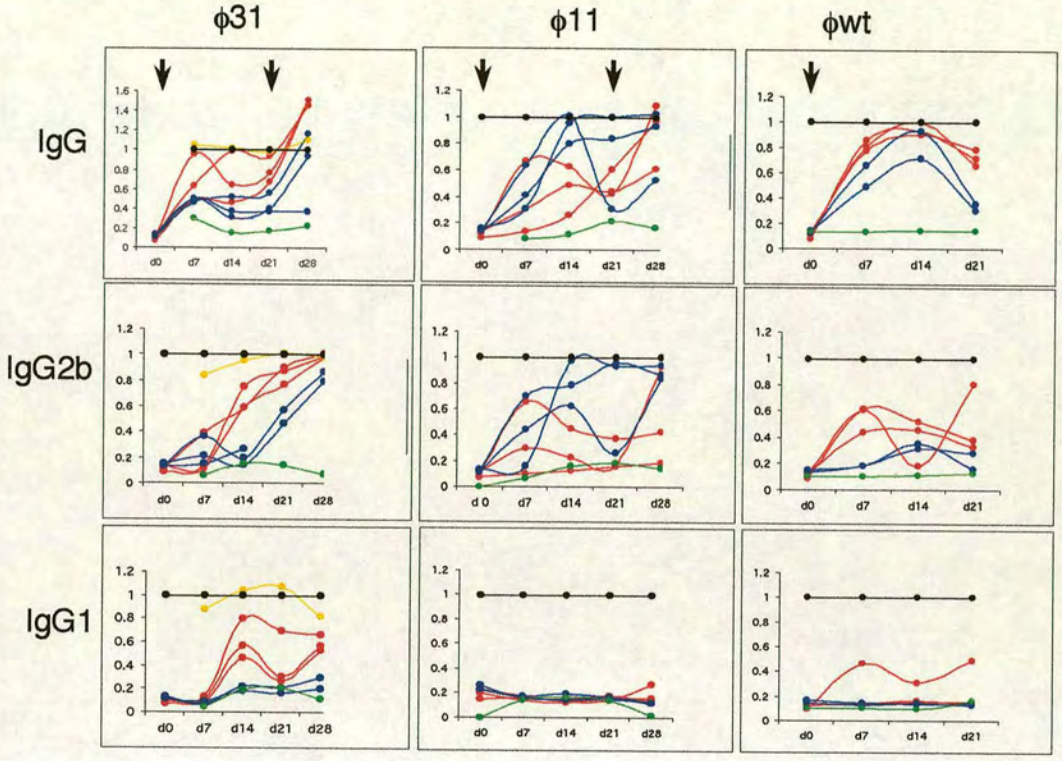
Mice received between 2 and $3x10^{6}$ 3.83 Id $^{+}$ B cells, a small number of negative cells to match the contamination level in the positive sorted cells, or no cells. 24 hr later mice were immunised s.c. in the hind leg with either of ϕ31, ϕ11

or ϕwt emulsified in CFA, they were boosted s.c. in the back on day 21. Blood samples were taken weekly for analysis of serum antibody responses and spleens harvested on day 28 for FACS analysis of the composition of transferred cells. Antibody responses of all IgG subtypes were measured by ELISA at each of 5 time points (Figure 5.9).

All immunised mice produced an IgG response to ϕ31 ϕ11 and ϕwt, even those that received negative cells or no cells, although in these cases the titres were lower (Figure 5.9). This IgG response is comprised of IgG2b and IgG3 isotypes. (Anti-IgG2a did not work in this assay). We propose that in this system the phage induces a TI response leading to IgG2b and IgG3 production by host CD40 deficient cells as in a TD response only CD40 positive cells should provide a class switched response.

In this experiment, IgG1 is only detectable in mice that received 3.83 transferred cells and were immunised with ϕ31. Mice that received 3.83 cells and were immunised with either ϕ 11 or ϕ wt do not make IgG1. In this case IgG1 is the only subclass not made by host $CD40^{-/-}$ cells. We had thought that we might be able to use IgG1 production as an indicator of the response of transferred cells. However, following further investigation, it became apparent that some CD40 deficient mice are able to make IgG1 responses to phage. Therefore measuring IgG1 production as a read out of the response of transferred cells is not robust enough and we are unable to make conclusions about the nature of the response of transferred cells to the phage in this system.

Figure 5.9 Relative titres of the response to phage



Mice received either ~2x10^6 3.83 positive cells (red) or sorted negative cells (blue). There are at least 3 mice per group. Mice were immunised 24hr later with one of the three phage, boosted on day 21 and blood samples taken weekly. Serum ELISAs for the mimotope were performed for IgG, IgG2b and IgG1. Titres are shown relative to the response of a phage immunised BALB/c mouse (black). The 3.83 response to ϕ31 is shown in yellow and the negative control is in green. From left to right are the responses of mice immunised with ϕ31, ϕ11 and ϕwt respectively. From top to bottom are IgG, IgG2b and IgG1 responses respectively.
Arrows indicate primary immunisations and boosts.

5.9 Mice that received 3.83-RAG cells make antibody of all IgG subclasses

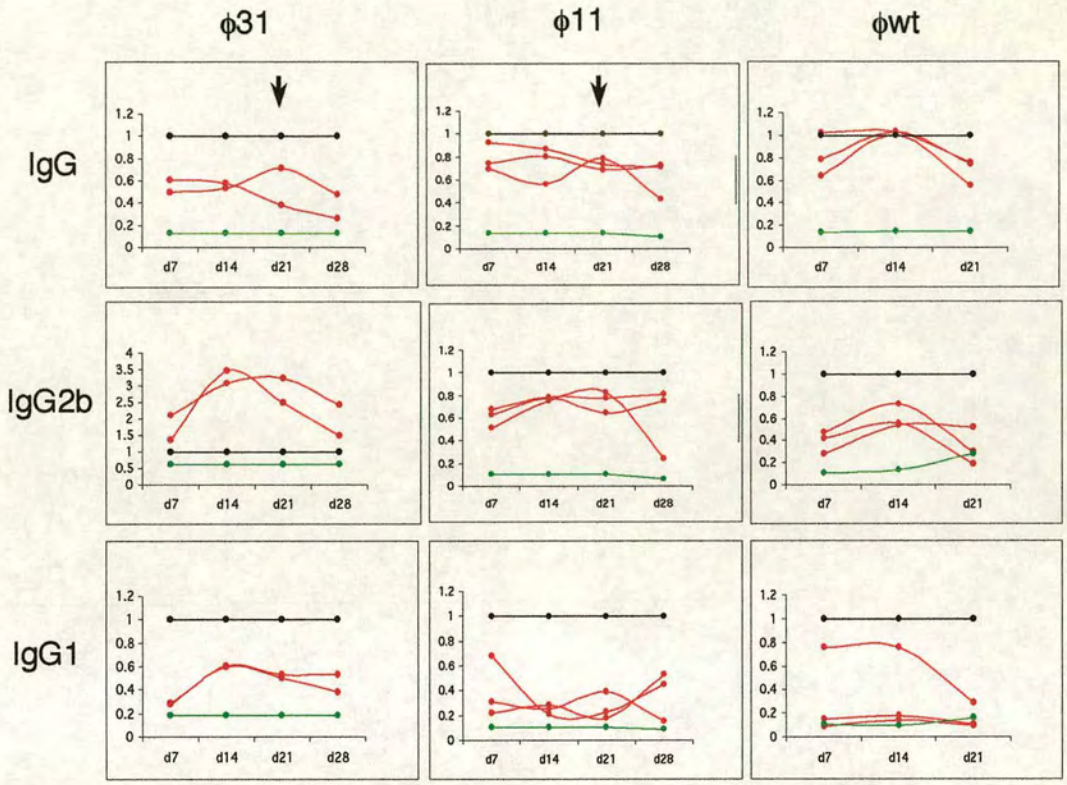
To make a comparison between cells that can undergo rearrangement of their BCR and those that cannot, $5x10^6$ 3.83 RAG cells were transferred to chimaeras and mice immunised 24hr later with one of the phage emulsified in CFA. Serum responses were measured by isotype specific ELISA. Almost all mice, except 2, made antigen specific IgG1 (Figure 5.10), this does not come from transferred cells as 3.83RAG cells do not make antibody responses to ϕ11 or ϕwt (not shown). This response must come from host CD40 deficient B cells possibly responding in a TI fashion (see section 5.13).

In the QM system, host cells did not contribute to the response to NP-OVA indicating that this result was specific to some component of the 3.83 experimental system. A thorough investigation of this result follows in section 5.11, examining the possibilities of the source of this response focussing on differences between this experiment and the successful QM experiment.

5.10 Transferred cells can be detected in the spleen after 4 weeks.

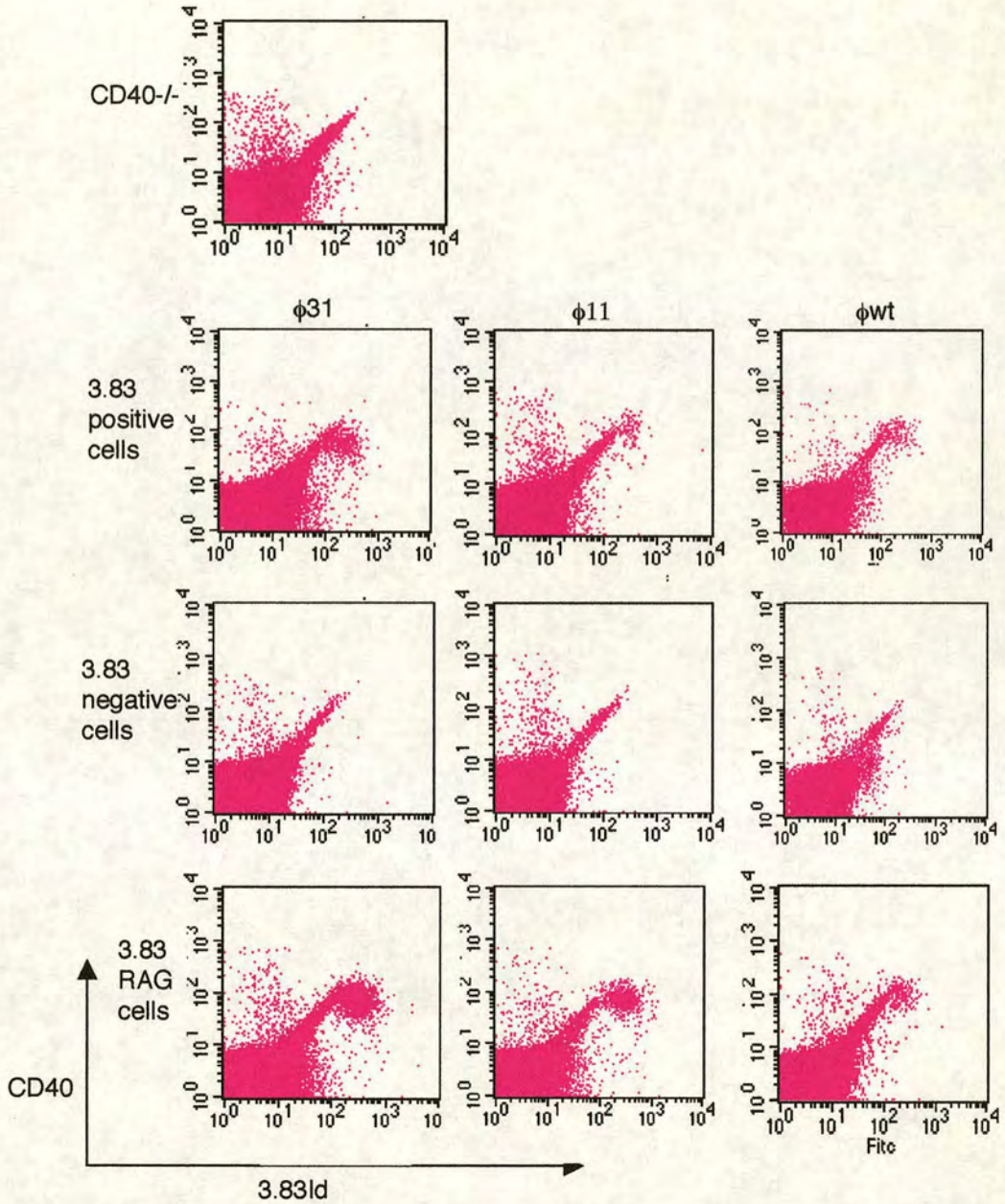
On day 28 post cell transfer, mice were sacrificed and splenocytes stained with antibodies to B220, CD40 and the 3.83 Id to detect the presence of transferred cells. Gating on $B220^{hi}$ cells to eliminate DC, B cells were analysed for CD40 and Id expression. Unlike host cells, transferred cells express CD40 and consequently can be segregated allowing analysis of Id expression (Figure 5.11). However, background CD40 staining and the presence of autofluorescent cells complicate enumeration of Id^+ and negative cells.

Figure 5.10 Responses of mice that received 3.83 RAG cells



Mice received 5x10^6 3.83 RAG cells (red). The negative control is in green. There are 2 (ϕ31) or 3 (ϕ11, ϕwt) mice per group. Mice were immunised 24hr later with one of the three phage, boosted on day 21 and blood samples taken weekly. Serum ELISAs for the mimotope were performed for IgG, IgG2b and IgG1. Titres are shown relative to response of a phage immunised BALB/c mouse (black). From left to right are the responses of mice immunised with ϕ31, ϕ11 and ϕwt respectively. From top to bottom are IgG, IgG2b and IgG1 responses respectively Arrows indicate boosts.

Figure 5.11 Transferred cells can be detected on day 28



On day 28 post cell transfer spleens were harvested and splenocytes stained with antibodies to B220, CD40 and the 3.83 Id. Plots above are gated on live lymphocytes and B220 high cells to exclude $CD40^+$ DC. From top to bottom are CD40-/- control mouse, mice that received 3.83 positive cells, negative cells, or 3.83 $RAG^{-/-}$ cells respectively. From left to right are mice immunised with ϕ31, ϕ11 and ϕwt respectively. Mice shown are representative of 3 mice per group

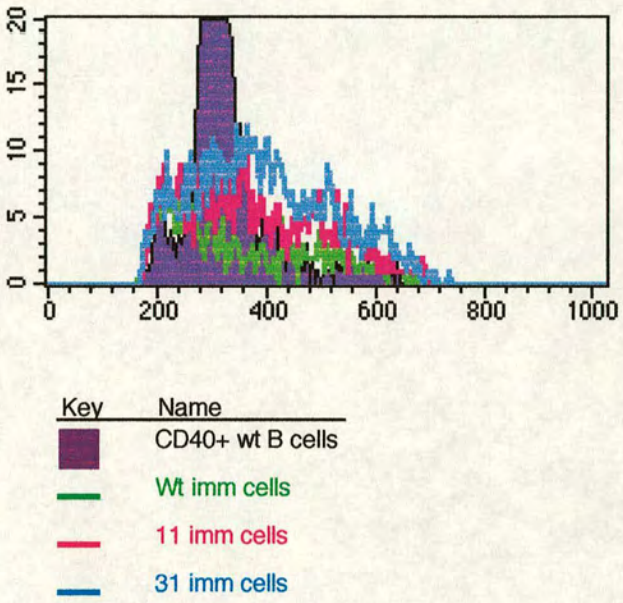
Despite this we see no evidence for expansion of Id$^-$ cells in this system. This may reflect the absence of receptor revision.

Activation of transferred cells was analysed by gating on CD40 positive cells and analysing forward scatter profiles (Figure 5.12). The Forward scatter profiles represent the hierarchy of binding with cells from ϕ31 immunised mice being largest followed by those from ϕ 11 and ϕ wt cells indicating that transferred cell were activated by antigen in this system.

5.11 μMT mice on a BALB/c background but not on a C57BL/6 background make serum IgG

Although we became aware that the source of IgG in this system was from TI class switched CD40$^{-/-}$ B cells, initially other sources were investigated. Firstly the μMT BALB/c mice were investigated. The QM experiment had been performed on the C57BL/6 background and host mice had not produced any IgG. As the μMT BALB/c mice had been newly generated for this experiment we checked the authenticity of these mice. Despite confirming the absence of B cells by flow cytometry we also measured the serum Ig levels in these mice. μMT mice on BALB/c and C57BL/6 background were immunised i.p. with 100μg of alum precipitated DNP-KLH with 10^9 *Bordetella pertussis*, mice were boosted on day 10 and antigen specific IgG production measured on day 14. None of the mice had any DNP specific antibodies, phage immunised μMT BALB/c also failed to produce an antigen specific response.

Figure5.12 Forward scatter profiles of transferred cells



Gating on $CD40^+$ cells, forward scatter profiles of cells in mice immunised with the 3 phage were compared. As a comparison $CD40^+$ B cells from an unimmunised wt mice were also analysed.
In purple is the forward scatter profile of B cells from a wt mouse, overlayed with cells transferred to a ϕ31 immunised mouse (blue), ϕ11 immunised mouse(pink) and ϕwt immunised mouse (green). Mice shown are representative of 3 per group

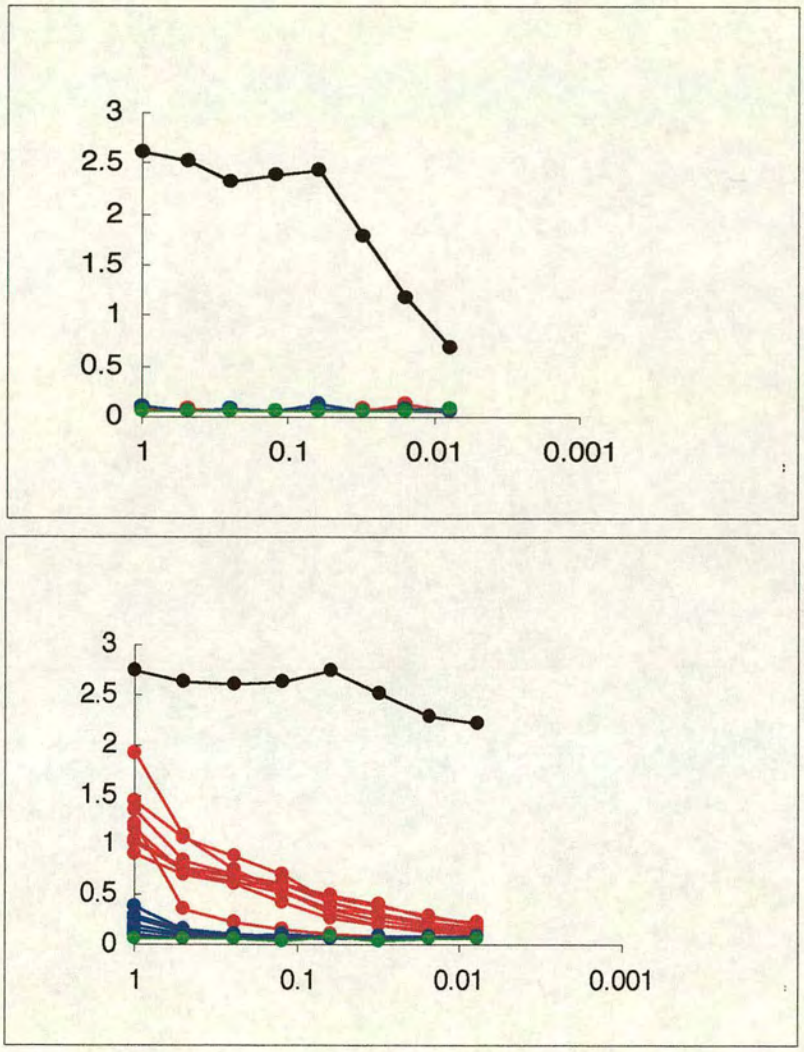
However, μMTs on the BALB/c background did have significant levels of total serum IgG but not IgM (Figure 5.13). This result was obtained at the same time as reports were made at the Immunology conference at Les Embiez. Rajewsky and co workers reported a phenomenon that occurs when μMT mice, are backcrossed to BALB/c. Due to differences in the genetic background there is a leak in the developmental block that halts B cell development and the BALB/c mice are able to produce IgG expressing plasma cells.

5.12 μMT serum IgG does not account for the IgG detected in this system

Given that μMT mice can produce IgG, albeit not antigen specific, we thought that in the context of a chimaera, μMT B cells might be able to account for the phage specific response detected by ELISA. We could think of 3 reasons for this:

1) μMT T cells, which have not been exposed to B cells, have been shown to have different characteristics to normal T cells (202, 203). They might lack the ability to provide the help required for B cells to make an antigen specific response. To assess this, $1x10^7$ T cells (> 99.5% pure) were transferred to μMT BALB/c mice to provide a source of normal T cells. Mice were immunised and boosted with DNP–KLH to see if the secretion of antigen specific antibody could be restored. No antigen specific IgM or G response was detected (not shown).

Figure 5.13 IgG production by μMT BALB/c mice



μMT Balb/c and μMt C57BL/6 mice were immunised ip with DNP-KLH alum and boosted on day 10 before analysis of serum antibody levels on day 14. Although no antigen specific antibody was detected (**A**)Total serum IgG was present in the BALB/c μMt mice, but not in the C57BL/6 μMt mice(**B**).
In both figures μMT Balb/c mice are represented by red lines and μMT C57BL/6 mice by blue lines, positive and negative controls are black and green respectively. There are 10 mice per group

2) B cells in the μMT mouse might differentiate into plasma cells due to the absence of other B cells. This phenomenon was discovered when B cells were transferred to lymphopeonic animals and may result from pressure to make protective antibodies (182). A similar finding has been reported in a mouse with an inducible mutation in the RAG gene (184). Plasma cells, being terminally differentiated are unlikely to be able to produce a *de novo* antigen specific response. In the presence of other B cells the leaky μMT B cells, might be arrested at naïve B cell stage and be able to provide an antigen specific response. To assess this, $6x10^6$ $CD40^{-/-}$ splenocytes were transferred to μMT BALB/c mice. In this case immunisation with DNP-KLH was performed one week later to allow potential leaky B cells to build up in the spleen. Again, although IgM was detected from the transferred $CD40^{-/-}$ B cells, no antigen specific IgG was produced (not shown).

3) IgM, which is lacking in μMT B cells is known to aid IgG production (204) through the production of immune complexes, IgM produced by CD40 deficient B cells in the chimaeric host, might induce the production of antigen specific IgG by μMT B cells. 150μl of serum from an immune $CD40^{-/-}$ mouse was transferred to mice, as a source of IgM to aid the IgG response. Mice were immunised with DNP-KLH, but again no antigen specific IgG response was detected (not shown).

Despite the fact that μMT/BALB/c mice have serum IgG they fail to make antigen specific responses even in the presence of normal T cells, $CD40^{-/-}$

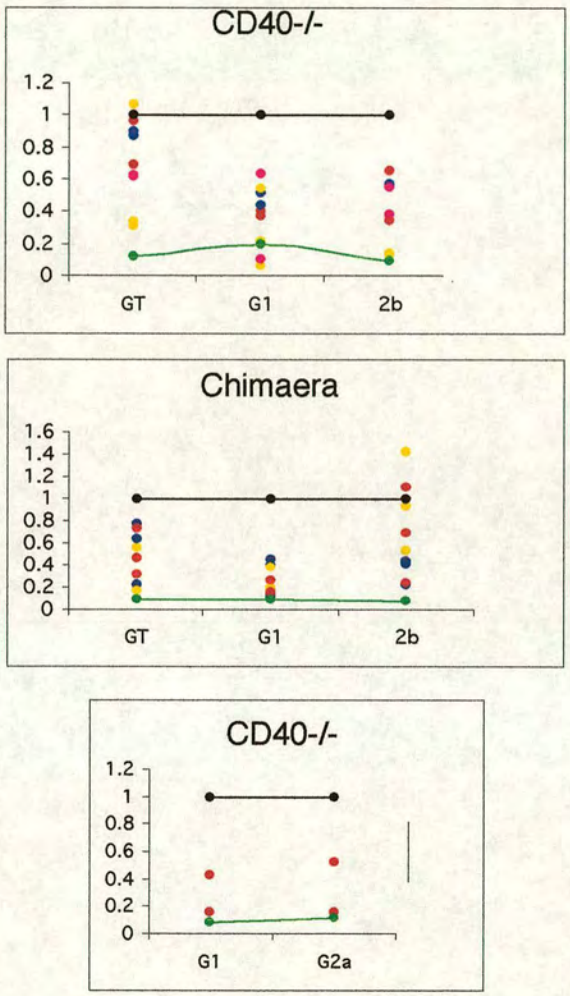
B cells and serum IgM, we conclude that these mice are not the source of antigen specific IgG in our system.

5.13 Immunisation with phage induces a T independent response resulting in the production of all antibody isotypes.

Having ruled out the μMT BALB/c mice as a source of antigen specific IgG in this system, further investigation was performed into the nature of the response induced by the phage antigen. A panel of $CD40^{-/-}$ mice and $\mu MT/CD40^{-/-}$ BALB/c chimaeras were immunised with all three phage to determine the nature of the IgG response induced (Figure 5.14). $\mu MT/CD40^{-/-}$ C57BL/6 chimaeras were also immunised with the wt phage. All mice made antigen specific IgG2b and IgG3, however, some mice also produced IgG1. The IgG1 response proved very inconsistent with only about 1 in 2 mice making a response, however we believe that this IgG1 response, produced by CD40 deficient B cells accounts for the IgG1 detected in the adoptive transfer system.

Due to the formation of GCs in phage immunised BALB/c mice and in chimaeras with transferred 3.83 cells we had thought that a TD response was generated. But it appears that there is also a TI aspect to the phage response. To confirm this result 3.83 RAG mice were immunised with ϕ 31 to see if they produced IgG1. These mice have no T cells and should not make class switched responses to TD antigens, however class switched antibodies of all types were detected, indicating a TI response.

Figure 5.14 Response to phage in CD40$^{-/-}$ mice and μMT/CD40$^{-/-}$ BALB/c chimaeras



CD40$^{-/-}$ (top) and μMT CD40$^{-/-}$ BALB/c chimaeras (middle) were immunised with the 3 phage and antigen specific serum antibody levels measured by mimotope ELISA. Titres relative to the day 14 response of a phage immunised BALB/c mouse are shown as dots for IgG, IgG1 and IgG2b from left to right. Yellow dots represent ϕ31 immunised mice, red dots ϕ11, blue dots ϕwt, pink 3.83 RAG $^{-/-}$ mice immunised with ϕ31, black and green lines represent BALB/c and negative controls respectively

The bottom plot shows IgG1 and IgG2a responses in CD40$^{-/-}$ Balb/c mice as measured by anti IgHa antibodies. Mice are p11 immunised.

We then questioned whether CFA, which contains bacterial antigens, might be responsible for the production of IgG by CD40 deficient B cells. To investigate this $CD40^{-/-}$ mice were immunised with myelin oligodendricyte glycoprotein (Mog) in CFA. IgG was not produced ruling out CFA as the cause of the IgG detected. BALB/c mice immunised with phage in PBS instead of CFA also produced IgG of all types and had GCs.

To confirm that what we were detecting was not an artefact of the complex mimotope ELISA system $CD40^{-/-}$ BALB/c mice were immunised and anti allotype subclass ELISAs performed. We had feared that the ELISAs, due to their inverted orientation, had lost specificity for isotype and that there was now cross reactivity between antibodies. Anti IgH^a antibodies, however are unlikely to cross react as they are mouse anti-mouse antibodies and the antigen on each subclass is different. The same results were obtained with IgG1 being detected in some of these mice. This leads us to the conclusion that phage can induce antigen specific IgG1 in $CD40^{-/-}$ mice. Given that the basis of our detection strategy was IgG production we do not feel that we can attribute the response detected solely to transferred cells.

5.14 Development of a system to allow distinction of transferred cells on the basis of expression of allotype marked antibody

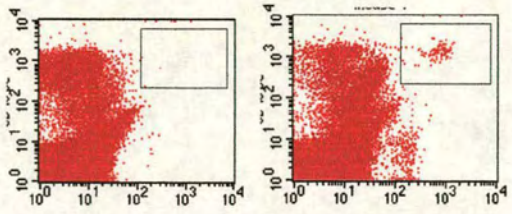
We had decided to use a chimaeric system, which allowed the detection of the response of transferred cells due their IgG response. Given that this is not possible due to the production of antigen specific IgG by CD40 deficient B cells

we developed another system which allows the distinction of the response of transferred cells due to their production of IgH^a antibodies. 3.83 mice express antibody of the IgH^a allotype and are backcrossed to BALB/c, CB20 mice are on a BALB/c background but carry a congenic heavy chain of the IgH^b allotype. This means that the response of transferred cells can be detected using anti IgH^a antibodies in ELISAs.

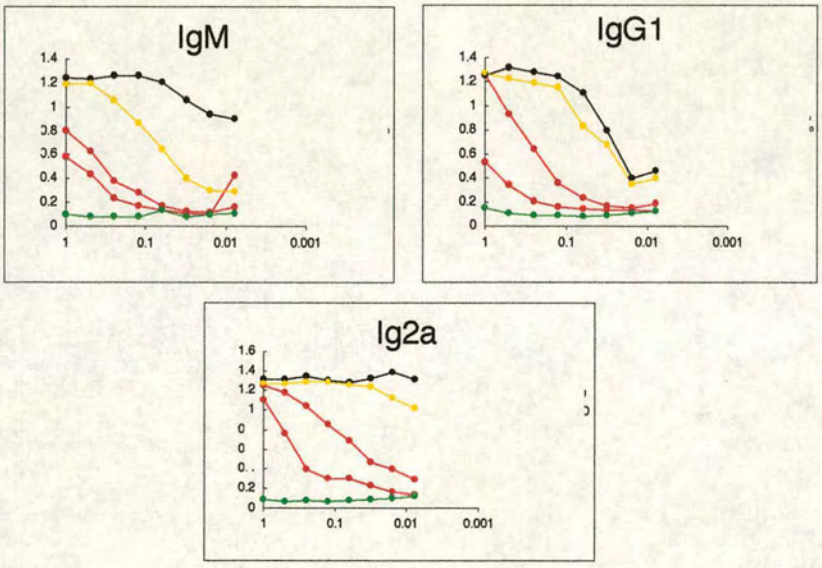
It was determined that for transferred cells to survive and divide, host mice need to be irradiated for 10min. However the same level of cell division of transferred CFSE labelled cells was not detected. Despite detecting ELISA responses in preliminary experiments (Figure 5.15), in two repeated experiments, although cells were detected, ELISA responses to phage were not. This system remains to be properly optimised.

Figure 5.15 CB20 system.

A



B



A CB20 mice irradiated for 10min (R) or unirradiated (L)received $3x10^6$ sorted 3.83 Id^+ cells and were immunisd with ϕ31. On day 14 splenocytes were analysed by FACS for the presence of transferred cells.
B IgM, IgG1 and IgG2a responses were measure by ELISA on day 14. Balck lines represent BALB/c responses, yellow 3.83, green negative control. Red lines represent the responses of 2 irradiated mice.

Discussion

Background

Receptor revision has been detected in splenic B cells by Nemazee and co-workers in the 3.83 mouse (137). It was initiated only in response to antigen recognised with low affinity by the BCR and not by high affinity or non-binding antigen. In the 3.83 transgenic mouse, secondary rearrangement results in the rearrangement of endogenous loci that are not normally expressed due to phenotypic exclusion by the pre-rearranged transgene. Rearrangements in the endogenous antibody light chain gene were detected by expression of the lambda light chain; the transgene encodes a kappa light chain. *De novo* V(D)J recombination was proven by the detection of lambda excision products by PCR in mice immunised with low affinity antigen only.

It has been suggested that this secondary V(D)J recombination of peripheral B cell might contribute to affinity maturation of the antibody response (157). Our experiment to test whether mature B cells can rearrange their receptors and subsequently gain affinity for poorly bound antigen was based on the findings of Hertz *et al.* (137).

In this chapter the transfer of a pure population of 3.83 B cells to CD40 $^{-/-}$ /μMT mixed bone marrow chimaeras is described. This was done so that any affinity matured IgG response detected could be attributed to transferred cells alone. This has not been possible in experiments performed in intact mice as all mice possessing BCRs encoded by transgenes or site directed Ig insertions have

small populations of B cells with non-transgenic specificities that might be responsible for the response detected.

The adoptive transfer strategy selected had proven successful in allowing the distinction of the response of transferred QM B cells (described in chapter 4). However, as the QM mice only possess a targeted heavy chain they require RAG expression to rearrange the endogenous light chain and create functional antibody. This means that affinity matured responses can be caused either by somatic hypermutation or receptor revision. 3.83 KI mice were crossed onto a RAG deficient background ruling out the contribution of receptor revision as secondary rearrangements are not possible in RAG deficient mice. We planned to compare the responses of transferred 3.83 KI cells, which can both mutate and revise their BCRs, with transferred 3.83 KI/RAG cells, which are only able to undergo mutation. This should allow the dissection of the contribution of both of these processes to affinity maturation. In using mice possessing the 3.83 receptor we also have the advantage of being able to use antigens of known affinity for the BCR. The low affinity antigen (ϕ11) has been shown to induce receptor rearrangement whereas the high affinity (ϕ31) and non-binding antigens (ϕwt) do not.

Firstly, as in the QM system, viability and division of transferred cells was confirmed by analysis of CFSE labelled cells after transfer to adoptive hosts. The formation of GC was also confirmed by immunofluorescent analysis of spleen sections, indicating the activation of transferred cells and their recruitment into the immune response.

Development of mimotope ELISAs

The development of an ELISA system to detect serum antibody responses to mimotopes expressed on the surface of phage was critical to the success of this experiment. The recombinant phage used to immunise mice in this system express 12 or 15 amino acid insertions in the pIII gene. This protein is present in 4 copies on the surface of recombinant M13 phage. Mimotope ELISAs for the detection of serum antibody responses have not been previously documented and is a significant new development in this project.

Access to the mimotope appears to be hindered when phage are fixed on the surface of polystyrene plates used for ELISAs. Detection of serum antibody specific for the mimotopes requires that phage is free floating. This means that serum antibodies have to be fixed to the plate using anti mouse isotype antibodies, phage is then bound by any mimotope specific antibodies present in the serum. Bound phage is subsequently detected with a polyclonal rabbit anti M13 phage antibody and an anti rabbit secondary antibody conjugated to HRP.

T independence of the response to phage

Once the detection system had been developed, serum antibodies to phage mimotopes could be detected in chimaeras. This led to the discovery of an unforeseen problem; M13 phage elicit a TI response in this system that results in the secretion of all antibodies isotypes. This makes distinction of the response of host CD40 deficient cells from the response of transferred cells impossible as we rely on CD40 being required for class switching in a TD response.

The recombinant M13 phage employed in this experiment has been reported to behave as a TI-2 antigen if the phage were crosslinked with anti-

phage antibodies (205). Polysaccharide antigens on the surface of bacteria are examples of TI-2 antigens; they possess multiple repeating epitopes that can activate B cells in an antigen specific fashion in the absence of cognate interaction with T cells. This distinguishes TI-2 antigens from TI-1 antigens exemplified by bacterial LPS, that induce a polyclonal, B cell response (39). The PIII protein is present in 4 repeats, which may explain its ability to induce TI-2 responses, although normally a valency of 10 or more is required.

Phage antigens, however, also initiate TD responses *in vitro* in the presence of anti-CD40 antibody (201). We have also discovered that BALB/c mice immunised with phage in either CFA or PBS elicited normal GC formation. Also, 3.83 cells transferred to chimaeras form plentiful GCs. This is a classic indicator of a TD response, therefore we thought that in the presence of T cells a TD response was elicited. T I-2 antigens can elicit class switched responses of all isotypes in the absence of CD40 (44). In the chimaera system both CD40 deficient and transferred CD40 sufficient B cells are present. The phage activated the former in a TI fashion and the latter in a TD fashion and the response elicited from these 2 subsets cannot be distinguished on the basis of isotype as had been planned.

IgG production by μMT BALB/c mice

We can now attribute the IgG response to phage in chimaeras to a TI response by host, CD40 deficient cells. Initially we had thought that μMT/ BALB/c mice, which reconstitute the chimaeras, might have been responsible. These mice had been newly generated and we feared an error in breeding was responsible. Although this was not the case μMT mice crossed onto a BALB/c background

did possess significant levels of serum IgG but not IgM. The production of antigen specific IgG was not detected. We investigated whether, in the context of CD40 deficient B cells, normal T cells or serum IgM present in bone marrow chimaeras, these cells might behave differently and produce the antigen specific IgG we were detecting. μMT BALB/c mice were supplemented with $CD40^{-/-}$ B cells, T cells from a normal mouse or serum IgM from an immune mouse. None of these factors allowed the production of antigen specific antibody in this system, further elucidating this interesting phenomenon.

We know that the IgG detected in μMT BALB/c mice is caused by the presence of plasma cells but not normal B cells. The fact that an antigen specific response cannot be induced is probably due to the fact that these cells are terminally differentiated. To make an antigen specific TD response, B cells need to interact with T cells in B cell follicles, some of them then proliferate and form GC, and others form plasma cells and secrete high levels of antigen specific antibody. The plasma cells detected by Rajewsky and co-workers probably were not selected in antigen specific manner.

B cells transferred to RAG deficient hosts quickly secrete high levels of IgM and serum levels approach normal (182). μMT mice have no membrane μ gene but the gene for secreted IgM and other isotypes is still present, however they still fail to secrete IgM. The serum IgG detected, however, is probably caused by a similar phenomenon. Class switching to IgG is normally a TD phenomenon, however in the absence of the μ gene a small number of B cells appear to be able to rearrange the Ig locus so that a functional antibody of the G

isotype is formed. Rearrangement of a functional heavy chain gene is required for cells to pass from the early pro B cells stage and undergo positive selection.

It is unknown why μMT mice on the BALB/c background and not μMT mice on the C57BL/6 background can undergo this phenomenon. However, it has been previously reported that Ig-μ–deficient/LPR mice on the C57/BL6 background do secrete IgG but not IgM (206). LPR mice lack functional FAS expression, which is important in the control of activated mature cells. It is postulated that lack of functional FAS may allow maturation of defective B cells in μMT/*lpr* mice accounting for the detection of serum Ig. It may be that μMT BALB/c mice do not regulate the development of B cells as tightly as μMT C57BL/6 mice, this could be due to heterogeneity in FAS function or expression or may be caused by something else.

The fact that the addition of normal T cells or immune serum IgM do not allow antigen specific IgG production adds strength to the argument that this is due to the terminal differentiation of these cells. We might, however have expected that the addition of other B cells would allow the production of antigen specific IgG. If the μMT B cells only become plasma cells due to the absence of other B cells as is the case in transfers to RAG deficient hosts, we might expect that in the presence of B cells and serum IgM from these cells, that differentiation would be halted at a stage where leaky cells could respond to antigen. Either this is not the case or the design of our small experiment did not allow it. One week after transfer of CD40 deficient B cells mice were immunised with DNP – KLH, this may not have allowed sufficient time for IgG expressing B cells from μMT mice to come out of the bone marrow. We do not know what

the frequency of the rearrangement event is, which allows cells to express surface IgG and undergo positive selection in the bone marrow.

No evidence for expansion of Id negative cells

Despite the fact that we were unable to discern whether affinity maturation occurred in this system due to an unfroseen TI response, we were still able to detect transferred cell after 28 days. Anti-Id staining, although complicated by high background from the anti CD40 antibody and autofluorescent cells, gave us no reason to believe that receptor revision had occurred in this system. Numbers of Id negative cell were not above background in any of the mice investigated.

Hybridomas were generated from spleen cells of all mice in this experiment and analysis of these for gene rearrangement by sequencing will give conclusive evidence of the presence of absence of rearrangements in transferred cells, however, this could not be performed in the allotted time.

Development of an allotype transfer system.

We had selected an adoptive transfer system, which allowed the distinction of the response of transferred cells on the basis of class switched antibody production. Due to the unusual properties of the phage antigen this proved impossible. A final system was developed to resolve this problem. CB20 mice have an MHC $H2^d$ background, which makes them compatible with BALB/c mice, but express an IgH^b allotype. In 3.83 mice the transgene bears the IgH^a allotype, allowing the response of these cells to be detected with allotype specific antibodies.

Although CB20 mice have the advantage of expressing a different allotype, transfers to these mice were unreliable. In some cases grafts did not survive, this is known to be a problem when transferring to immunocompetent hosts (186). Although some transfers were successful and IgH^a antibodies were detected from transferred 3.83 cells, we failed to get consistent responses from transferred cells, even if grafts were detected by FACS.

Conclusion

Despite our considerable efforts to determine whether 3.83 cells are able to undergo secondary V(D)J recombination, and provide affinity matured responses to low affinity antigen, we were unable to do so in this system. We were unable to distinguish the antibody response of transferred cells from that of the host due to the production of IgG in a TI fashion by CD40 deficient B cells. However, analysis of the Id expression of transferred cells by FACS gave no evidence for the expansion of cells that had lost Id expression by receptor revision. This could be due to the fact that the phage induced a polyclonal response in all cells in the mouse and therefore there was not a strong selective pressure for the expansion of revised cells. However, this may give some insight into that fact that this phenomenon may only be readily detectible if selective pressure is strong.

One of the initial arguments against receptor editing in the bone marrow was that it was an artefact of transgenic systems, which could only be detected if no other B cells were able to respond. This was addressed by Retter and Nemazee (28) by examining the frequency of functional rearrangements that had been inactivated by recombining sequence (RS) rearrangement. If the

antibody rearrangement in the bone marrow results in the production of non-functional antibody it is normal to rearrange the other allele. However if functional antibody was generated the only reason to rearrange the other allele is if the antibody had an auto reactive specificity. The functional antibody is shut down by recombination with the down stream RS sequence. In normal B cells 47% of RS-inactivated VκJκ joins were in frame, suggesting that receptor editing occurs at a high frequency in normal mice.

A similar argument could be wielded for the case of receptor revision; that it is only detectible in the case of mice with a limited B cell repertoire. However, heavy chain V gene replacement has been documented in rheumatoid arthritis synovial tissue B lymphocytes in humans (153) and in normal B lymphocytes from human tonsils (155). It has been reported that the frequency of such events is low (<3%) and unlikely to play a major role in the shaping of the repertoire as revised B cells are most likely counter selected (156). It is now believed that secondary rearrangements may contribute to the development of autoreactive specificities, rather than useful specificities as we hypothesised (150, 154). However, one recent study has documented the contribution of peripheral light chain rearrangement to the generation of high affinity antibodies and stated that receptor revision can contribute to affinity maturation (176). This was in a mouse where the diversity of the repertoire was limited due to the presence of a site directed transgene specific for the hapten NP. However, the fact remains that revised B cells can be detected in normal systems where the repertoire is already diverse.

Receptor revision may be a rare event in organisms with a normal repertoire. Its effect may only be noticeable in cases where the either the

repertoire is limited by the presence of a transgenic receptor, where selection of a rare cell of another specificity is strong or in cases of abnormalities in B cell regulation; patients with autoimmune disorders. We were unable to distinguish the response of transferred cells due to an unforeseen TI response. However, the presence of cells with altered specificities was not detected by FACS implying that receptor revision does not play a major role in a situation where other cells are capable of providing a suitable immune response.

Conclusions

The aim of this study was 2 fold: to investigate triggers of somatic hypermutation and to investigate the contribution of receptor revision to affinity maturation.

We have initiated somatic hypermutation *in vitro*, using defined molecular stimuli. Stimulation of naïve B cells with antibodies to sIg, CD40 and CD38 induced high level mutation with hallmarks of that induced *in vivo*. Studies *in vivo* using mice deficient in CD40 and CD38, confirmed the importance of stimulation through CD40 for the induction of mutation. This is the first formal proof of the absence of somatic hypermutation in CD40 deficient mice. The role of CD38 has been shown to be non-specific and its importance is most likely as a powerful B cell mitogen, allowing fixation of the mutations introduced *in vitro* during cell division.

Our investigations into the role of receptor revision in affinity maturation proved less conclusive. In 3 chapters, I have outlined: 1) the development of an adoptive transfer system to facilitate detection of the affinity maturation of adoptively transferred transgenic B cells; 2) Experiments investigating affinity maturation using B cells from the QM mouse, which undergo secondary rearrangements at the heavy chain locus; 3) Experiments using B cells from the 3.83KI mouse, which undergo receptor revision at the light chain locus. These studies had varying degrees of success due to problems encountered at late stages in the experiments.

Previous studies into receptor revision have not investigated its role in affinity maturation. We have employed an adoptive transfer system where only

newly introduced changes in the BCR of a pure population of B cells will facilitate affinity maturation. The response to antigens recognised with low affinity by 2 different transgenic BCRs has been investigated in the same manner.

The development of a suitable host for the adoptive transfer of transgenic B cells was critical for the success of this experiment. The formation of GC by transgenic cells is critical for their affinity maturation as GC are the site of both somatic hypermutation and selection of high affinity mutants. Peripheral RAG expression has also been associated with GC (130, 133, 134) intimating that they are the location of secondary V(D)J rearrangement. We use a novel chimaeric mouse as an adoptive host in which we can be assured that only adoptively transferred cells can form GC and undergo affinity maturation therein. This system allows the selective expansion of transferred cells in a CD40 dependent fashion, giving them an advantage over host CD40 deficient cells. The presence of the host cells provides a physiological environment for this to occur.

In the 2 experimental systems described different problems were encountered making it difficult to come to firm conclusions about receptor revision and affinity maturation. In the QM system the use of well-characterised TD antigens resulted in an IgG response, which could be attributed to transferred cells alone. This assay demonstrated a small affinity matured response to TNP, an antigen to which no binding was detected in the primary response. However, we are unable to attribute this to receptor revision alone, as somatic hypermutation may be responsible. Analysis of Id expression by FACS should have elucidated whether cells had undergone receptor revision resulting in loss of Id expression. However, all transferred QM cells displayed

diminished levels of Id expression making it difficult to discern whether these cells had lost Id expression.

In the 3.83 system, similar analysis of Id expression by transferred transgenic cells demonstrated no evidence for the expansion of Id negative cells due to receptor revision or otherwise. However, due to the complex nature of the immune response elicited by the phage antigen both TD and TI antibody responses were detected. Class switching in response to TD antigens in this system would have identified the response of donor (3.83KI) cells, as host cells were CD40 deficient and cannot class switch. However, the unanticipated TI response could have given rise to IgG from either donor or host cells.

More information could be obtained from the QM system if clearer data could be obtained about the Id expression of transferred cells by FACS. The 3.83 mice, however, proffer a better-defined system in which to investigate receptor revision and affinity maturation. We have crossed these mice to a RAG deficient background providing a source of B cells in which secondary rearrangements are impossible. This allows a direct comparison to be made between B cells that can or cannot undergo this process, allowing us to distinguish the contribution of somatic hypermutation from that of receptor revision. We have already developed an Ig allotype detection system for the analysis of the response of 3.83 cells transferred to Ig congenic hosts. This circumvents the problem caused by the detection of a TI response in chimaeras as it does not rely on the production of class switched isotypes. However, this system still needs to be optimised.

Future analysis of hybridomas generated from both QM and 3.83 systems will also enable the makeup of the Ig locus to be examined for secondary

rearrangements and point mutations, revealing precisely the contribution of both of these processes to the affinity maturation of Ig responses.

Materials and Methods

Animals

All mice were maintained under specific pathogen free conditions at the Ashworth Laboratories, University of Edinburgh. C57BL/6, BALB/c and CBA mice were bred in house. $CD40^{-/-}$ (44) and μMT mice (207) on both BALB/c and C57BL/6 backgrounds were also bred in house and maintained in filter topped cages. $RAG^{-/-}$ 2 deficient mice (7) on both backgrounds were maintained in isolators. CB20 mice were a gift from Dr Neil Mabbott (Institute of Animal Health, Edinburgh University, UK). QM (178) mice were kindly donated by Dr Peter Lane (University of Birmingham, UK) with the permission of Dr. Mattias Wabl (UCSF, San Francisco). 3.83KI mice (177) were the gift of Dr Roberta Pelanda (Denver, Colorado, USA). ELK mice (89) were kindly provided by Dr. Michael Neuberger (LMB, Cambridge, UK). CD38 knockout mice (B6x129 F2) were provided by Dr. Maureen Howard (Anergen, Redwood City, CA). Mice were used at 8-12 weeks of age and sex matched as closely as possible.

Protein Antigens

Ovalbumin (OVA, Sigma, Dorset, UK)), bovine serum albumin (BSA, Sigma) and keyhole limpet haemocyanin (KLH, Calbiochem, San Diego, California, USA) were used as protein antigens. These were coupled to dinititrophenyl by reacting with 23μl of dinitrofluorobenzene (DNFB, Sigma) solution in oil in 0.1M sodium borate buffer, pH 8.4 (Sigma) per mg of protein. OVA and BSA were coupled to nitrophenyl (NP) by reacting with 200μg of NP-O-Suc (Biosearch Technologies Inc. Novato, CA, USA.) in dimethylformamide (Sigma), in 0.2M

carbonate/bicarbonate buffer pH9.0. Following coupling hapten–protein conjugates were dialysed extensively against PBS to remove free hapten. TNP-OVA and TNP-BSA were purchased from Biosearch technologies. Phenyloxazolone Ph-OX-CSA was a gift from Dr. Harry White (UCL, London , UK). Alum precipitation of antigens was performed in 4.5% potassium alum (Sigma) solution.

Preparation of recombinant M13 phage to be used as antigen

XL1-Blue bacteria, which contain the F plasmid (possessing a tetracycline resistance gene) required for phage infection, were grown overnight in the presence of tetracycline (Sigma). Bacteria were infected with phage by inoculating a 1/100 dilution of an overnight culture with phage from a single plaque. Following a 3hr incubation cultures were then grown overnight in 300ml 2xYT culture medium (Sigma). The next day bacteria were removed by centrifugation for 15min at 8000rpm. Phage was precipitated from the remaining supernatant on ice for 1hr by the addition of polyethylene glycol (final concentration 5%) (PEG, Sigma) and NaCl (0.5M). Precipitated phage was centrifuged for 15min at 8000 and PEG precipitated a second time. Following a final centrifugation phage particles were washed in PBS to remove bacterial debris and remaining PEG before quantitation by spectophotometry.

Immunisations

Mice were immunised i.p. with 100μg of alum precipitated protein antigen with 10^9 killed *Bordetella Pertussis* (Calbiochem). Boosts where performed were with 100μg of soluble antigen except for QM transfer experiments where boosts were

the same as primary immunisations. Phage immunisations were performed sub cutaneously (s.c.) in the hind flank with 100μg of phage emulsified in CFA (Sigma), boosts if required were s.c. in the back.

Cell preparations

Single cell suspensions of spleens and lymph nodes were prepared in Hank's balanced salt solution (HBSS, Sigma) supplemented with 2% foetal calf serum (FCS, Labtech, Andover, Massachusetts, USA), by pressing lymphoid tissue through nylon gauze with forceps. These were further prepared by flushing through a 25 gauge needle (Becton Dickinson, San Jose, CA, USA). Red blood cells were removed by incubation in red blood cell lysing solution (Sigma) and washed in PBS.

Bone marrow cells were flushed from femurs with a syringe containing HBSS. Peripheral blood mononuclear cells were prepared by layering blood samples collected from mice in heparin over lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada). White cells were removed using a Pasteur pipette and washed in PBS.

Analysis of cell division by CFSE

Cells were loaded with the dye CFSE (Molecular Probes Inc., Eugene, Oregon, USA) at a concentration of 10nM for 10 minutes at 37°C. The number of cell divisions undergone by cells was measured at the appropriate time using FACS analysis (Becton Dickinson, FACS Calibur running CellQuest).

B cell stimulations

B cells were prepared by negative selection using anti-CD43 MACS microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturers instructions. $1x10^6$ B-cells/well were plated out in 24 well tissue culture plates (Costar, Corning, New York, USA) in Iscove's Modified Dulbecco's Medium (IMDM, Sigma) supplemented with 5% FCS (Labtech), 2mM L-Glutamine (Gibco-BRL, Paisley, Scotland), 50mM 2-mercapto-1ethanol (2-ME,Sigma) and penicillin-streptomycin (50mg/ml, Sigma). The cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. The following stimuli were added, singly or in combination: LPS (*S. typhosa* 0901) at 5μg/ml (Difco Labs, Detroit, Michigan, USA) anti-CD38 supernatant at 1/200, (NIMR-5 (167) provided by Dr. Michael Parkhouse, IAH, Pirbright, Surrey, UK), anti-CD40 (FGK-45 (208)) at 10μg/ml and anti-kappa light chain (187.1) at 10μg/ml. Supernatants from alloreactive Th2 clone (209) produced as described previously were used at a 1/10 dilution. The cells were harvested after 7 days

PCR amplifications and sequencing

Total RNA was prepared using RNAzol B (AMS Biotechnology, Oxon. UK) and then cDNA was made using a Promega Reverse Transcription kit (Promega Corporation, Madison, Wisconsin, USA) containing all the reagents for synthesis of single-stranded cDNA; both according to manufacturers instructions. The VκOx1 transgene (600 bp) was amplified from the cDNA using the following primers:

EK16 - 5'-GCCGGAATTCCCAGAGGACAAATTGTTC-3' (VκOx1)

RtCk- 5′-GCCCGGATCCGACGGGTGAGGA-3′ (ratCκ) and the following protocol:

95°C for 5 min. and then 30 cycles of 95°C 1 min, 53°C 1 min, 72°C 1min and then a 72°C extension for 10 minutes. The PCR product was purified using QIAquick PCR purification kit (Qiagen, Chatsworth, CA), ligated into P-Gem-T easy vector (Promega) before transforming competent JM109 bacteria. Colonies were picked and minipreps made (QIAprep spin plasmid miniprep kit; Qiagen). Colonies containing the correct insert were selected by digestion with Apa1 and Pst1(New England Biolabs, Beverly, Massachusetts USA). Sequencing was performed by MWG Biotech (Ebersberg, Germany).

For analysis of the J-C intron flanking the 3′ border of V_HJ558 genes, the method utilised was essentially that described by Jolly *et al.* (161). Briefly, genomic DNA was isolated using a QIAprep tissue kit (Qiagen). The J_H4 intron was amplified using a primer specific for FR 3 of J_H558 family of V_H genes (5′-GGAATTCGCCTGACATCTGAGGACTCTGC-3′) and the 3′ end of the intronic enhancer (5′-GACTAGTCCTCTCCAGTTTCGGCTGAA-3′) using a 2 stage protocol: 10 cycles of 94 °C 15 seconds, 64 °C-55 °C (reducing 1 °C per cycle) 15 seconds, 72 °C 4 minutes and then 25 cycles of 94 °C 15 seconds, 63 °C 15 seconds and 72 °C 4 minutes. The J_H4 rearrangement gives a product of 1200bp and this was excised, extracted from the gel using QexII (Qiagen,), cloned into pBluescript at the EcoRV site and sequenced using a primer specific for J_H4 (5′-TATGCTATGGACTACTGG-3′). The polymerase error rate was calculated by sequencing 20 clones derived after RT-PCR of message from a hybridoma expressing germline VκOx1. This error rate was 1/1200.

Cell proliferation assays

B cells were prepared by negative selection using anti-CD43 MACS microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturers instructions. $2x10^5$ B-cells/well were plated out in 96 well tissue culture plates (Costar, Corning, New York, USA) in IMDM (Sigma) supplemented with 5% FCS (Labtech), 2mM L-Glutamine (Gibco-BRL, Paisley, Scotland), 50mM 2-ME (Sigma) and penicillin-streptomycin (50mg/ml, Sigma). The cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. The following stimuli were added, singly: LPS (*S. typhosa* 0901) at 5μg/ml (Difco Labs), anti-CD38 supernatant at 1/200, (NIMR-5 (167) provided by Dr. Michael Parkhouse, IAH, Pirbright, Surrey, UK), purified anti-CD38 at 10μg/ml, anti-CD40 (FGK-45 (208)) at 10μg/ml , IL-4 supernatant at 1/100 (from cell line X63). Polymyxin B was used at a range of concentrations up to 100μg/ml.

After 48 hrs in culture 1 μCi ^3H thymidine in 10 μl complete medium was added to each well, and plates were incubated for 16-18 hr at 37°C prior to harvesting and counting using a Top Count Microplate Scintillation Counter (Canberra Packard).

Cell sorting

B cells isolated from mouse spleens by MACS were washed and adjusted to $1x10^8$/ml in HBSS (Sigma) supplemented with 2% FCS (Labtech). Cells were then incubated at 4°C for 20 min with FITC conjugated anti-Id antibody (for QM mice clone R2.438.8 at 1.5μg/ml (178) supplied by Dr Peter Lane, Birmingham; for 3.83 clone 54.1 at 1.6 μg/ml (15) supplied by Dr David Nemazee, Scripps Institute, San diego, USA) and PE-conjugated anti-B220 monoclonal antibody

(clone RA3-6B2 Becton Dickinson at 0.4 μg/ml) Id positive and negative cells were sorted using a FACS Vantage flow cytometer (Becton Dickinson running CellQuest software). Sorted cells were then washed and resuspended in PBS. Purity was assessed using a FACS Calibur Flow cytometer and cells were counted using trypan blue (Sigma) and a Neubauer haemocytometer (Marienfeld, Lauda-Königshofen, Germany).

In vitro *generation of bone marrow derived dendritic cells*

DCs were derived from bone marrow cells according to the procedure developed by *Inaba et al.* (210). In brief, bone marrow cells were cultivated in RPMI (Sigma) supplemented with 10% FCS (Labtech), 2mM L-Glutamine (Gibco-BRL), penicillin-streptomycin (50mg/ml, Sigma), sodium pyruvate (1mM, Sigma), granulocyte-monocyte colony stimulating factor supernatant 1/20 (GM-CSF; cell line X63 (211)). On the third day, non-adherent cells were washed away and remaining cells were cultivated in the same culture medium supplemented with 1.5% mouse serum instead of FCS. An additional wash was performed on day 6. By day 7 cultures typically contained >90% DC and were pulsed with antigen at 100μg/ml for 3 hours before being harvest and washed in preparation for injection into mice.

Generation of chimaeric hosts

Recipient mice were lethally irradiated (dose dependent on strain used see results chapters 3, 4 and 5; irradiation delivered from caesium source) on day 0. On day 1 bone marrow cells were flushed from femurs with a syringe containing HBSS (Sigma) and passed through a 25 gauge needle (Becton

Dickinson) to generate a single suspension. They were depleted of T cells by negative selection using anti-Thy-1(T24)-biotin, streptavidin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and separation on a MACS magnetic column (Miltenyi Biotec) according to the manufacturers instructions. Cells of donor origin were mixed at the required ratio and $5x10^6$ bone marrow cells injected i.v. into the irradiated recipients. The chimaeras were left for 8 weeks to allow reconstitution of the immune system before use.

To construct bone marrow chimaeras in which gene expression was restricted to defined cell populations, the host haemopoietic system had to be completely replaced with donor bone marrow cells. To ensure that there was no out growth or retention of host cells fully allogeneic chimaeras were tested 8 weeks after reconstitution. When bone marrow from BALB/c mice ($H2^d$) is transferred to lethally irradiated C57BL/6 mice ($H2^b$) greater that 99% of the peripheral B cells and DC are of donor origin as assessed by FACS staining with anti-CD19 (clone ID3, Becton Dickinson) and anti-CD11c (clone HL3 Becton Dickinson) and with antibodies to IA^b (clone AF6-120.1) and IA^d (clone MKD6). Furthermore essentially all of the DC generated *in vitro* from culture of bone marrow cells and non- adherent bone marrow precursors, are of the donor origin.

The bone marrow chimaera used in these experiments has CD40 expression on DC but not B cells. Irradiated mice were reconstituted with bone marrow from 2 sources: 80% from μMT and 20% from $CD40^{-/-}$ mice. To ensure that all the B cells were CD40 deficient B cells from these mice were stimulated with anti-CD40 (clone FGK-45) and proliferation measured. No proliferative

response was detected from these cells. This result was confirmed by FACS staining of splenocytes with anti-CD40 antibody (clone 3.23 Becton Dickinson) and anti IgM (polyclonal goat anti mouse, Southern Biotech, Birmingham, Alabama, USA)

Adoptive transfers

For transfers of QM and 3.83 B cells, between 1 and $3x10^6$ sort purified Id positive cells were transferred to adoptive hosts. To account for the small amount of contamination in the Id positive population the requisite number of Id negative cells were transferred to control mice. $5x10^6$ cells were used in adoptive transfers of 3.83RAG cells. In preliminary experiments to assess survival of transferred cells $1x10^7$ splenocytes were transferred.
Mice were immunised 24 hours after cell transfer.

Measurement of antigen specific serum Ig by ELISA

Blood collected from tail veins of mice was allowed to coagulate at room temperature, then centrifuged twice and serum collected. 96 well microtitre plates (Dynex Technologies Inc, Chantilly, Virginia, USA) were coated with antigen at 50μg/ml in 0.1M carbonate/bicarbonate buffer, pH 9.6 overnight at 4^0C. Plates were washed with PBS and blocked with PBS 1% BSA or PBS 1% dried milk powder for one hr at room temperature (RT). Plates were washed and serum added in 2 fold dilutions in PBS 1% BSA for 2hr at RT. Plates were washed in PBS 0.05%Tween 20 (Sigma) and incubated with alkaline phosphatase (AP) conjugated polyclonal goat, anti-mouse IgM or IgG or IgG subtypes (Southern Biotech) in PBS 1% BSA for 2hr. Following a final wash

plates were developed with p-nitrophenyl phosphate, disodium salt (pNPP, Southern Biotech) in substrate buffer (10% diethanolamine, 5% $MgCl_26H_2O$ pH 9.8). The optical density was measured at 405nm.

Mimotope ELISAs

96 well microtitre plates (Dynex Technologies Inc, Chantilly, Virginia, USA) were coated with isotype specific antibody at 10μg/ml in 0.1M carbonate/bicarbonate buffer, pH 9.6 overnight at 4^0C. Plates were washed with PBS and blocked with PBS 2.5% dried milk powder (mPBS) for one hr at room temperature (RT). Plates were washed and serum added in 2 fold dilutions in 1% mPBS for 2hr at RT. Plates were washed in PBS 0.05%Tween 20 and incubated with phage at 0.1mg/ml in 1% mPBS for 2 hrs. Free phage was washed away and bound phage detected with polyclonal rabbit anti-Fd (Sigma) for 1hr at RT. Following a wash, anti-rabbit horseradish peroxidase (Dako, Glostrup, Denmark) was added for one hr at RT. Plates were developed using 10%w/v Tetramethylbenzidine (TMB) substrate (Sigma) diluted in substrate buffer (0.05M Sodium Phosphate, 0.025M Citrate pH 5) with 0.03% Hydrogen peroxide. The reaction was stopped after 3-5 min with 2M sulphuric acid and the optical density measured at 450nm.

Histology

Spleens were harvested, embedded in Cryo-M-Bed embedding compound (Bright Instrument Company, Huntingdon, UK) and frozen at -80^0C. Frozen sections (5μM thick) were fixed in cold acetone and dried extensively. Sections were stained with FITC conjugated anti-Thy1 (clone T24) for T cells and Texas

Red conjugated anti IgM for B cells (Southern Biotech). GC were stained with either FITC or biotin conjugated PNA (Vector Labs. Burlingame, California, USA). Primary stains were for 2 hr, where required secondary stains were performed with streptavidin Texas Red (Southern Biotech) for 1 hr. After staining, sections were washed extensively in PBS before mounting and analysis using an Olympus BX50 microscope under reflected fluorescent light. Images were captured using a Hamamatsu digital camera and Openlab image analysis software (Improvision, Coventry, UK

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Appendix

Signals That Initiate Somatic Hypermutation of B Cells In Vitro[1]

Sigridur Bergthorsdottir,* Aoife Gallagher,[2]* Sandra Jainandunsing,* Debra Cockayne,† James Sutton,* Tomas Leanderson,‡ and David Gray[2,3]*

Somatic hypermutation is initiated as B lymphocytes proliferate in germinal centers. The signals that switch on the mutation process are unknown. We have derived an in vitro system to define signals that will initiate mutation in normal, naive splenic B cells. We find that three signals are required to allow detection of somatic mutation in vitro; these are anti-Ig, anti-CD40, and anti-CD38. If any one of these is omitted, mutation remains off. We show that CD40 is obligatory in vivo, as CD40 knockout mice exhibit no Ag-driven mutation. In contrast, CD38 is not, as CD38 knockout mice mutate normally. We believe that, in vitro, CD38, in combination with other stimuli, drives extensive cell division, allowing the detection of mutated sequences. However, in germinal centers in vivo, proliferative activity is instigated by a different molecule. This is the first demonstration of the initiation of hypermutation in vitro with normal splenic B cells using defined stimuli. *The Journal of Immunology,* **2001, 166: 2228–2234.**

Affinity maturation of the Ab response occurs largely as a result of selection of the Ig V gene products that have undergone a process of somatic hypermutation. The vast majority of late primary and secondary response Abs exhibit changes from the germline V sequence (1, 2), and most memory B cells carry somatically mutated B cell receptors (BCR)[4] (3). In generating the repertoire for affinity selection to act upon, somatic hypermutation forms an integral part of the process of memory B cell development, and the two processes seem to be intimately linked, both occurring in the same anatomical site. Germinal centers (GC) are necessary for the generation of memory B cells (4) and are sites in which B cell hypermutation is switched on (5). Under normal, physiological circumstances GC appear to be the only sites of mutation (6), although, if pushed to the extreme by repeated immunization with enormous amounts of Ag, mutations can be detected in lymphotoxin-$\alpha^{-/-}$ mice that lack GC (7).

B cell memory development, GC reaction, and somatic hypermutation occur only during responses to T-dependent Ags (generally proteins), suggesting that signals from T cells are necessary at some point in all of these processes. The exact molecular identity of the signals driving these processes is still unknown. We do not know what initiates the GC reaction, what drives GC B cell proliferation, or what causes differentiation within the GC (e.g., the transition of centroblast to centrocyte). CD40 is implicated in the first two functions as CD40 knockout mice develop no GC (8, 9); however, its role in GC induction may be an indirect one as rudimentary GC can be regenerated in these mice simply by injection of CD40-Ig (10). However, CD40 signals do seem to be crucial during the final stage of memory differentiation in GC; the final rescue of mutated GC B cells from apoptosis (11) and entry into the memory pool (12). Signals via the BCR are clearly also crucial, as without recognition and uptake of Ag the B cell would be unable to elicit T cell help. Whether the BCR signals generated by small protein Ags have any role to play in driving the cell through cell cycle is not known. Ag uptake and subsequent processing for presentation is important at two stages of the B cell response: the initiation and for the selection of mutants in the GC for survival in the memory pool (13). So we know that the processes involved in memory generation require signals via surface immunoglobulin and signals derived from T cells; indeed, we have shown that somatic mutation could be maintained (although not initiated) by culturing B cells with anti-Ig and helper T cells (14). This has since been supported by similar findings using human B cell lines (15, 16) and tonsillar B cells (17). The exact identity of these T cell-derived signals is still mysterious.

In this study we have set out to find the signals that initiate somatic mutation of B cells in vitro. To do this we have stimulated B cells from a transgenic mouse carrying a *VκOx1* gene with all of the upstream elements required to target somatic mutation to the V gene (18). As this Vκ is rearranged to a rat Cκ the transgene is readily identified, avoiding the problem of unequivocal identification of the germline equivalent of a mutated Vκ that is often difficult in normal mice due to close similarities within V gene families. B cells from these mice were stimulated and maintained in culture under a number of conditions. These included BCR cross-linking in combination with the various effector molecules of T cell help (i.e., CD40 ligand and cytokines). B cells stimulated using anti-Ig together with anti-CD40, and supernatants from Th2 clones did not accumulate somatic mutations in the *VκOx1* transgene. However, the replacement of cytokines with an Ab to CD38 led to the detection of somatic mutation in vitro.

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[4] Abbreviations used in this paper: BCR, B cell receptor(s); GC, germinal center(s).

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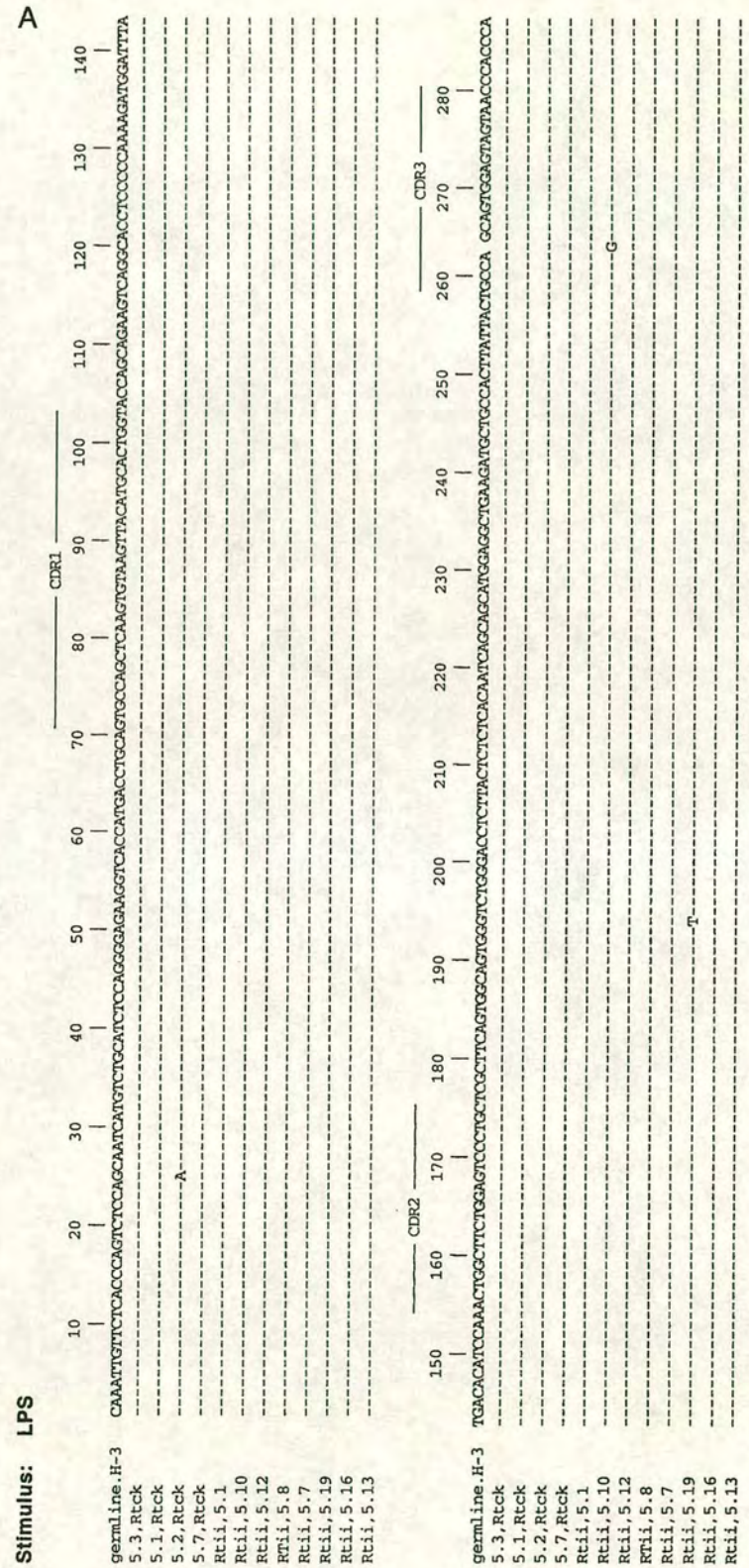

FIGURE 1. Mutation initiated in vitro. Sequences of the *VκOx1* transgene from B cells cultured with LPS (*A*) and anti-κ, anti-CD40, and anti-CD38 (*B*). The top line shows the germline sequence; only changes from the germline are indicated. Note that similar results were obtained in three other experiments.

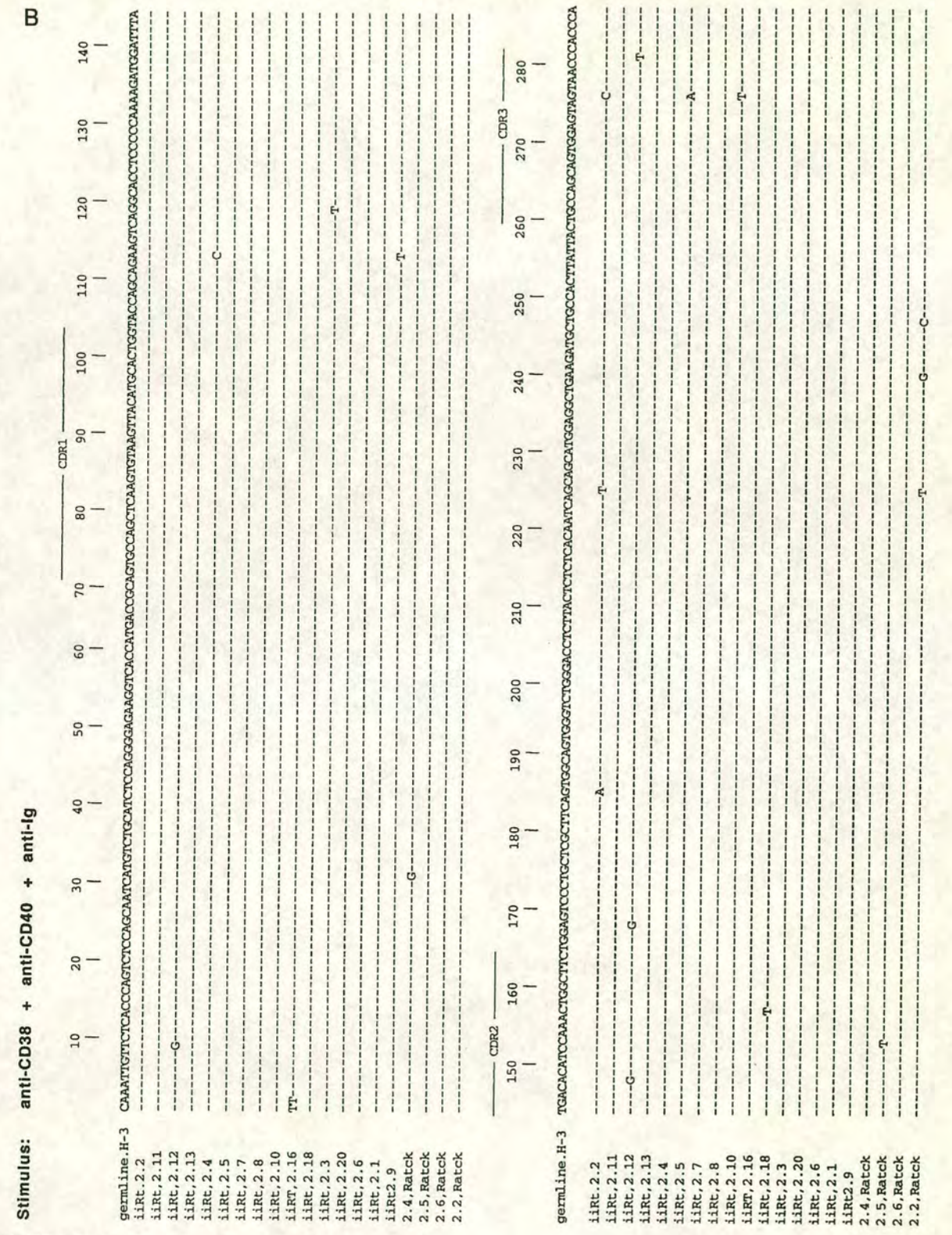

FIGURE 1. Continued.

Table I. *Frequency of somatic mutations in the* VκOx1 *transgenic in splenic B cells stimulated in vitro*

Stimulus[a]	No. of Sequences	No. of Mutated Sequences	No. of Mutations[c]	Frequency of Mutations[d]	p^e
Anti-CD38 + anti-CD40 + anti-Ig	20	9	20	1/290	0.016
Anti-CD38 + anti-CD40	15	6	6	1/725	0.73
Anti-CD38 + anti-Ig	14	4	6	1/676	0.52
Anti-CD40 + anti-Ig	22	9	10	1/627	0.56
Anti-CD38 + anti-CD40 + anti-Ig + Th2 supernatant	6	1	1	1/716	0.75
Anti-CD38	8	0	0	<1/2320	0.65
LPS	12	3	3	1/1140	–

[a] Cultures are the same as those from which the sequences in Fig. 1 were derived.
[b] Number of independent sequences used for analysis.
[c] Total number of mutations in all mutated sequences.
[d] Number of mutations in all sequences divided by the total number of base pairs sequenced.
[e] Probability using Fisher's exact test that the difference in mutation frequency between LPS and triple stimulus is due to chance.

Materials and Methods

Mice and immunizations

ELK mice, carrying a transgene incorporating *VκOx1* and upstream regulatory elements (18), were provided by Dr. Michael Neuberger (LMB, Cambridge, U.K.). CD38 knockout mice (B6 × 129 F_2) (19) were provided by Dr. Maureen Howard (Anergen, Redwood City, CA) and CD40 knockout mice (8), originally made by Dr. Hitoshi Kikutani (Osaka, Japan) have been maintained in our laboratory for some time and backcrossed to C57BL/6 for seven generations. Wild-type mice were C57BL/6 strain. All mice were bred and maintained under standard laboratory conditions in the animal facilities of Imperial College School of Medicine, Hammersmith Hospital and later at the Ashworth Laboratories, University of Edinburgh. Mice were used at 8–12 wk of age. Mice were immunized via the i.p. route with 100 μg of alum-precipitated phenyloxazolone-chicken serum albumin together with 10^9 killed *Bordetella pertussis* and then boosted 3 wk later with 100 μg of soluble phenyloxazolone-chicken serum albumin.

B cell stimulations

In early experiments B cells were prepared from spleens by the depletion of T cells using IgM Abs against Thy1, CD8, and CD4 and lysis with mouse-absorbed young rabbit complement (C-Six Diagnostics, Mequon, WI). Residual T cells were depleted using Dynabeads (Dynal, Oslo, Norway) coated with IgG Abs to the same surface markers (Abs and procedure are described in Ref. 20). In more recent experiments B cells have been prepared by negative selection using anti-CD43 MACS microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according the manufacturer's instructions and described in Skok et al. (21). Using both methods, B cell preps were routinely >95% pure with <1% contamination with $CD3^+$ T cells. One million B cells/well were plated out in 24-well tissue culture plates (Costar, Corning, NY) in IMDM supplemented with 5% FCS, 2 mM L-glutamine (Life Technologies, Paisley, Scotland), 50 mM 2-ME, and penicillin-streptomycin (50 mg/ml). The cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. The following stimuli were added singly or in combination: LPS (*Salmonella typhosa* 0901) at 5 μg/ml (Difco, Detroit, MI), anti-CD38 supernatant at 1:200 (NIMR-5, Ref. 22, provided by Dr. Michael Parkhouse; IAH, Pirbright, Surrey, U.K.), anti-CD40 (FGK-45, Ref. 23) at 10 μg/ml, and anti-κ light chain (187.1) at 10 μg/ml. Supernatants from alloreactive Th2 clone (20) produced as described previously were used at a 1:10 dilution. The cells were harvested after 7 days.

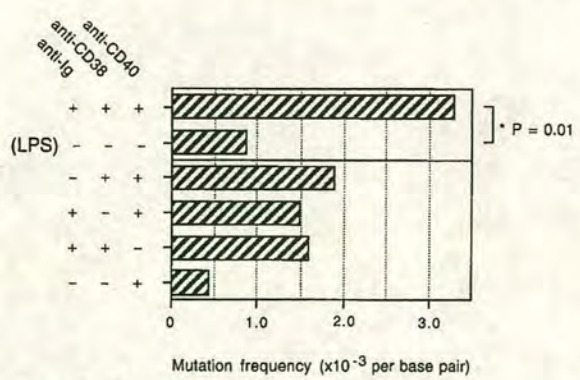

FIGURE 2. Mutation frequency in B cell cultures. The frequency of mutation obtained in the *VκOx1* transgene following 7-day culture of B cells with combinations of anti-κ (anti-Ig), anti-CD40, and anti-CD38. Only the combination of all three stimuli was statistically, significantly different from the LPS control (using Fisher's exact test). This result is representative of three other experiments

Analysis of cell division

The number of cell divisions undergone in culture was measured using FACS analysis (FACSCaliber running CellQuest; Becton Dickinson, Mountain View, CA) at appropriate times of cells loaded with the dye CFSE (Molecular Probes, Eugene, OR) at a concentration of 10 nM for 10 min at 37°C as previously described (24).

PCR amplifications and sequencing

Total RNA was prepared using RNAzol B (AMS Biotechnology, Oxon. U.K.), then cDNA was made using a Promega Reverse Transcription kit (Promega, Madison, WI) containing all the reagents for synthesis of single-stranded cDNA (both according to manufacturer's instructions). The *VκOx1* transgene (600 bp) was amplified from the cDNA using the following primers: EK16, 5′-GCCGGAATTCCCAGAGGACAAATTGT TC-3′ (*VKOx1*); and RtCk, 5′-GCCCGGATCCGACGGGTGAGGA-3′ (*ratCκ*); and the following protocol: 95°C for 5 min and then 30 cycles of 95°C for 1 min, 53°C for 1 min, 72°C for 1 min, and then a 72°C extension for 10 min. The PCR product was purified using a QIAquick PCR purification kit (Qiagen, Chatsworth, CA) and ligated into P-Gem-T easy vector (Promega) before transforming competent JM109 bacteria. Colonies were picked and minipreps made (QIAprep spin plasmid miniprep kit; Qiagen). Colonies containing the correct insert were selected by digestion with *ApaI* and *PstI*. Sequencing was performed by MWG Biotech (Ebersberg, Germany).

For analysis of the J-C intron flanking the 3′ border of V_HJ558 genes, the method used was essentially that described by Jolly et al. (25). Briefly, genomic DNA was isolated using a QIAprep tissue kit (Qiagen). The J_H4 intron was amplified using a primer specific for framework region 3 of J_H558 family of V_H genes (5′-GGAATTCGCCTGACATCTGAGGAC TCTGC-3′) and the 3′ end of the intronic enhancer (5′-GACTAGTC CTCTCCAGTTTCGGCTGAA-3′) using a two-stage protocol: 10 cycles of 94°C for 15 s, 64°C-55°C (reducing 1°C per cycle) for 15 s, 72°C for 4 min, and then 25 cycles of 94°C for 15 s, 63°C for 15 s, and 72°C for 4 min. The J_H4 rearrangement gives a product of 1200 bp; this was excised, extracted from the gel using QexII (Qiagen), cloned into pBluescript at the *Eco*RV site, and sequenced using a primer specific for J_H4 (5′-TATGC TATGGACTACTGG-3′). The polymerase error rate was calculated by sequencing 20 clones derived after RT-PCR of message from a hybridoma expressing germline *VκOx1*. This error rate was 1:1200.

Results and Discussion

Experimental system

All in vitro experiments were conducted with B cells from a transgenic mouse (ELK) expressing VκOx1 linked to a rat Cκ. The transgenic construct contains both the 3′ κ enhancer and the intron

Table II. *Summary of mutation frequencies obtained in four separate in vitro experiments*

Stimulus	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Anti-CD38 + anti-CD40 + anti-Ig	1/363[a] (8/10)[b]	1/410 (12/17)	1/399 (24/33)	1/290 (20/20)
Anti-CD38 + anti-CD40	ND[c]	ND	1/580 (5/10)	1/725 (6/15)
Anti-CD38 + anti-Ig	ND	1/653 (7/16)	1/967 (3/10)	1/676 (6/14)
Anti-CD40 + anti-Ig	1/580 (6/12)	1/628 (6/13)	1/773 (6/16)	1/627 (10/22)
Anti-CD38 + anti-CD40 + anti-Ig + Th2 supernatant	ND	ND	1/870 (2/6)	1/1716 (1/6)
LPS	1/1740 (2/12)	1/1450 (4/20)	1/1160 (5/20)	1/1140 (3/12)
p value[d]	0.015	0.02	0.02	0.016

[a] Frequency of mutation = number of mutations in all sequences divided by the total number of base pairs sequenced.
[b] Total number of mutations in the sequences analyzed/number of independent sequences analyzed.
[c] ND, Not done.
[d] Probability using Fisher's exact test that the difference in mutation frequency between LPS and triple stimulus is due to chance. The value for all other stimulations conditions are >0.4.

enhancer that together facilitate targeting of somatic mutation to the transgene (18). The gene product is expressed at the cell surface and is secreted into the serum; however, as far as this experiment is concerned, it is used as a passenger transgene in that we do not analyze Ag-driven responses. It has the advantage that we can readily identify the mutated transgene by amplifying with a PCR primer for the rat Cκ together with a VκOx1-specific primer. This is important as the unequivocal identification of mutated V genes in mice is complicated by the close homology within families and the possibility that not all the members of that family are yet cloned. Thus, splenic B cells were purified (>98%) and placed in culture under a variety of conditions for 7 days. The culture medium and stimuli were replenished after 4 days (see *Materials and Methods*). After harvesting cells, RNA was prepared and the *VκOx1* transgene was amplified by RT-PCR, cloned, and sequenced.

Initiation of somatic mutation in vitro

The stimuli used in the cultures were designed to mimic signals delivered to B cells during T-dependent responses, i.e., antigen (anti-Ig), CD40 ligand (anti-CD40), and cytokines (delivered as a mixture of Th2 cytokines contained in a supernatant from an activated Th2 clone). We also tested an Ab to CD38 that has strong mitogenic properties on mouse B cells (22). As previous studies had indicated that mutation did not occur during culture of B cells following LPS stimulation (26), we used LPS as a negative control. As demonstrated in Fig. 1*A*, the B cells cultured with LPS for 7 days exhibited no mutation of the *VκOx1* transgene above the *Taq* error rate (~1:1200). Somatic hypermutation was found under only one culture condition, containing three stimuli together: anti-Ig, anti-CD40, and anti-CD38. Fig. 1*B* shows a significant number of point mutations scattered through the *VκOx1* transgene from B cells cultured in this way. Approximately half (9 of 20) of the sequences analyzed contained mutations (see Table I), and most had two or more mutations per sequence. Both Table I and Fig. 2 indicate that the difference in mutation frequency between LPS (1:1140) and the triple stimulus (1:290) is statistically significant. Table I and Fig. 2 also show that mutation can only be initiated if all three stimuli are present; omission of any one results in loss of significant mutation. A very striking result is the observation that the addition of Th2 cytokines to the anti-Ig, anti-CD40, anti-CD38 culture inhibits the accumulation of somatic mutations, we think because they drive differentiation of B cells to plasma cells. In Table II we show data from four independent experiments that confirm that the triple stimulus of anti-Ig, anti-CD40, anti-CD38 reproducibly induces mutation in vitro. This culture condition is the only one tested in which the difference in mutation frequency compared with the LPS control, which could not occur due to chance, according to Fisher's exact test. The frequency of mutations observed with dual stimuli was greater than that in the LPS controls but did not reach statistical significance. For stimulation conditions involving anti-CD40 this increase was consistent in four independent experiments; for anti-Ig plus anti-CD38 the increase was seen in only two of the three experiments. This could indicate that CD40 signals are sufficient on their own to switch on mutation that is detectable only when other stimuli are present to enhance cellular proliferation.

Naive B cells have been stimulated to accumulate mutations in vitro previously either within a splenic fragment assay (27) or in coculture with an activated T cell clone (17). Neither of these studies revealed which signals were involved in the process. Here we identify three signals that can initiate mutation in vitro and may be crucial in vivo. It is interesting that CD40 was reported not to induce mutation in the study of Razanjaona et al. (17); however, the cultures reported in that study included CD40 ligand transfectants plus a mixture of cytokines. We also find no mutation when cytokines are added to our "mutation stimuli".

We have analyzed the mutations incorporated into the *VκOx1* transgene in B cells stimulated with anti-Ig, anti-CD40, anti-CD38 for hallmarks of somatic mutation. The point mutations detected are scattered throughout the V region, with more than half in the framework regions; however, there does seem to be an accumulation in complementarity-determining region 2 (Fig. 1*B*). This pattern of mutation in sequences from triple stimulation cultures was similar in four independent experiments (80 sequences). It may be that the complementarity-determining region targeting of mutations observed previously is related to antigenic selection, of which

Table III. *Nature of base substitutions observed in cultured B cells*

Mutations[a]	Observed (%)	Expected[b] (%)
Transitions		
Total	56.3	33.3
C→T	18.8	9.3
A→G	25.0	8.0
G→A	6.3	7.4
T→C	6.3	7.0
Transversions		
Total	43.7	66.6
A→T	12.5	8.0
G→T	25.0	7.4
G→C	6.3	7.4

[a] Mutations found in the nine mutated sequences derived form the anti-Ig, anti-CD40, anti-CD38 cultures.
[b] Expected number of mutations was calculated by multiplying the frequency of a particular nucleotide (mutation target) in the germline sequences by the total number of observed mutations and dividing this by 3 (as mutation to any of the other three nucleotides has the same theoretical probability).

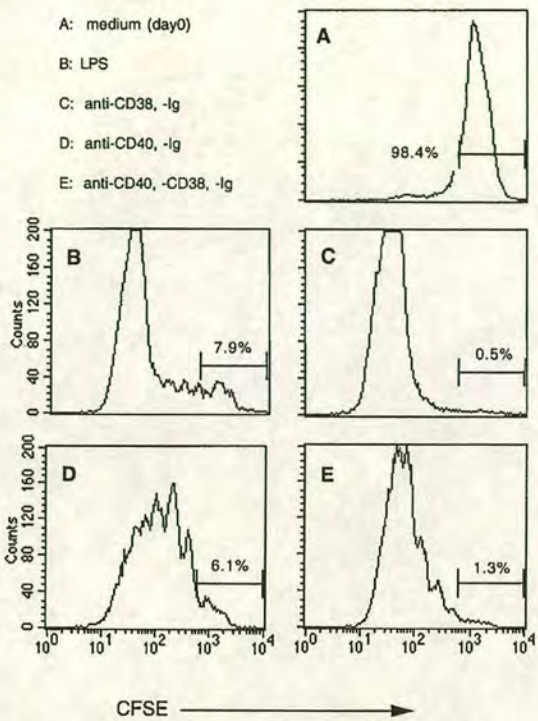

FIGURE 3. Proliferation of B cells in culture. Flow cytometric analysis of CFSE labeling of B cells cultured for 7 days with various stimuli (*A–E*, as indicated). These data are representative of four such experiments.

there is none in this system. As expected, for this reason, the ratio of the number of replacement mutations compared with the number silent mutations is little different from the predicted value (data not shown). Table III shows that there is an over-representation of transitions compared with transversions in the set of mutations from the triple stimulation culture. A preference for transitions is an intrinsic feature of the hypermutation machinery. Transitions should make up only 33% of the mutations; however, in this data set 56% of the mutations are transitions. We have to keep in mind that up to 25% of the mutations we see could be taq-induced (our taq error rate = 1:1200) and these would also be biased toward transitions. If we take away 25% of the observed transitions, the proportion of such mutations is, at 43%, still some 10% greater than the expected frequency. Some changes are particularly prevalent, such as C→T, A→G, and G→T; these have been noted previously in a study of mutation in a cell line (16). Mutation in the intrinsic mutational hotspots is not observed in the sequences illustrated here (Fig. 1); thus we see no mutation of the serine 26, 31, or 77 codons (28, 29). Moreover, these mutations were not observed in the larger data set (Table II) above the frequency expected by chance.

Calculation of the rate of mutation in this culture is in the range previously estimated for somatic hypermutation in vivo. The mutation frequency in the triple stimulation culture is 3.4×10^{-3} per base pair. We estimated, using the halving of CFSE fluorescence with each cell division (Fig. 3), that the cells in this culture pass through six generations. Thus the rate of mutation is 5.8×10^{-4} per base pair per generation. This is 10-fold lower than the maximum quoted rate in vivo of 5×10^{-3} (30). It has been argued that a rate of 3×10^{-4} per base pair per generation is more accurate. Most calculated figures are, in fact, below 10^{-4} (31, 32). It is also worth remembering that the rate varies throughout the response, such that it is low to start with and increases at the height of the GC (33). On this basis and given the fact that the in vitro culture may lack the optimal microenvironment, we are gratified that the mutation rate is of the correct order of magnitude.

Is mutation related to number of cell divisions?

CD38 might play one of two roles in the initiation of somatic mutation in vitro. First, it could provide a specific signal to start mutation or second, it could drive proliferative expansion of mutated cells to a level within the cultures at which they are readily detectable. We analyzed the proliferative activity of B cells stimulated in various ways by labeling the starting population with CFSE and counting the number of cell divisions (by halving of CFSE fluorescence) at the end of 7 days. Fig. 3*C* shows that anti-CD38 and anti-Ig cause the majority of cells to enter cell division and proceed to the maximum number of six divisions. This confirms what we already know (Table I and Fig. 2), that CD38 is not an "on switch" for somatic mutation, at least, not in the absence of CD40 signals, as even with maximal cell division no mutation is seen. Anti-CD38, in combination with anti-CD40 and anti-Ig, increases the number of cells proceeding through the maximum six divisions (compare Fig. 3, *D* and *E*) and as such may well be working to drive expansion of mutants to a detectable level. It should also be noted that, in the anti-CD38 stimulations, the majority of cells enter the cell cycle (98.7 vs 93.9%; Fig. 3, *D* and *E*), and so no subpopulation of B cells is left at the proliferative starting gate as happens with LPS or anti-CD40 (see Fig. 3, *B* and *D*). We have found no other stimulation conditions that have such a broad and powerful mitogenic/proliferative effect. Although we cannot rule out absolutely the possibility that some of the mutations we see in our cultures derive from the expansion of preexisting memory cells, the proliferation data suggest otherwise. Anti-CD38 plus anti-Ig seem to push most cells into cycle (Fig. 3*C*), but mutation is not significantly enhanced. We also have in excess of 40 ELK transgene sequences from splenic B cells, which

Table IV. *Frequency of somatic mutation sin the* V_H*J558/ J/C intron in immunized CD40 and CD38 knockout mice*

Mice/Immunizations	No. of Sequences[a]	No. of Mutated Sequences	No. of Mutations[b]	Frequency of Mutations[c]
Wild-type/nonimmune	13	12	24	1/4660
Wild-type/immune	11	8	53	1/169
$CD40^{-/-}$/immune	13	3	5	1/1820
$CD38^{-/-}$/immune	12	7	38	1/198

[a] Number of independent sequences used for analysis.
[b] Total number of mutation sin all mutated sequences.
[c] Number of mutations in all sequences divided by the total number of base pairs sequenced.

exhibit no evidence of mutation despite the presence of ongoing 'irrelevant' immune responses (e.g., splenic GCs) in the mice.

In relation to the cell proliferation it is clear that none of the sequences obtained from these cultures are clonally related, i.e., no sequential accumulation of mutation during culture could be detected. This is not unexpected given the cell input number and the relatively small sample size. We are currently trying to extend the useful life of the cultures in the hope of achieving a larger clone size and observing such clonal development. It might then also be possible to introduce selection into the system.

Somatic mutation in CD40 and CD38 knockout mice

To see whether CD40 and CD38 play a role in hypermutation in vivo we immunized CD40- and CD38-deficient mice (19) and analyzed the accumulation of mutations in their V regions. To do this we sequenced the J-C intron flanking the 3′ border of V_HJ558 genes in B cells from immunized mice, as described by Jolly et al. (25). The data are summarized in Table IV. As expected, in the light of the absence of GC in CD40$^{-/-}$ mice (8, 9), no somatic mutation was found. This may not be surprising, however, it is the first formal demonstration of impaired somatic mutation in these mice. The role of CD40 in the mutation process is obligatory in vitro and in vivo. The obvious interpretation is that CD40 directly initiates the mutation process by inducing expression of one of the components of the "mutator complex"; however, it might act indirectly following induction of other costimulatory receptors on the B cell surface. The latter possibility is indicated by the report that an EBV-negative Burkitt's lymphoma (BL2) mutates following anti-Ig stimulation and interaction with T cells, independently of CD40 (34).

In contrast, the J-C intron in B cells from CD38 knockout mice was heavily mutated. Our conclusion must be that CD38 is not necessary in vivo for the initiation or maintenance of somatic hypermutation. Although the proliferation it drives in vitro is crucial for the outgrowth and detection of somatic mutants, it appears that in vivo CD38 is not required for proliferation of mutating cells in GC, and that some other molecule performs this function. We cannot rule out that CD38 causes clonal expansion of selected cells following mutation, we have not analyzed the clone size of the mutated cells from CD38$^{-/-}$ mice compared with wild type.

Concluding remarks

Our data suggest that resting B cells require nothing other than Ag and CD40 ligand as a donor of T cell help to initiate the hypermutation process. We found that CD38 signaling was required in our in vitro model before mutations were evident, but its role was to enhance proliferation rather than to act as a specific on-switch: This pro-proliferative role for CD38 was dispensable in vivo. We propose that, in addition to CD40, another, still unidentified, molecular signal drives proliferation of centroblasts in GC.

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